CHARLES UNIVERSITY IN PRAGUE

Pharmaceutical faculty in Hradec Králové

Department of Pharmaceutical Botany and Ecology

Antioxidant and antiradical activity of selected species of Division Bryophyta

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Prague, 2007

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I declare that I created this diploma thesis by myself under the leadership of my supervisors and I used only the cited literature.

Prague, ……..

Samar J. Nabbout

1. Preface

This work was carried out at the Division of Pharmaceutical Biology, Faculty of Pharmacy, University of Helsinki, during September- December 2007. And at the Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague.

My sincere gratitude is due to Docent Into Laakso for his support, advices, help and positive attitude during my stay in University of Helsinki.

I wish to express my appreciation to my supervisors Prof. RNDr. Luděk Jahodář and Pharm.Dr Vladimír Chobot, PhD. for valuable ideas and advices.

My special thanks are due to Pharm.Dr. Lenka Kubicová, PhD, she has provided my valuable professional skills and I want to thank for the pleasant atmosphere in the laboratory.

Special thanks are due to Ph.D. Tuulikki Seppänen-Laakso, H.J. Damien Dorman and Keyvan Distmalchi.
I want to thank for those enjoyable moments outside the laboratory.

Finally, I reserve my warmest thanks to my parents, Hana and Jacques, for their patients, support and understanding during the course of my study.

List of abbreviations

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AHPA	Acid- hydrolysable phenolic acids
BHT	Butylated hydroxytoluene
BHPA	Alkaline-hydrolysable phenolic acids
DMSO	Dimethyl sulphoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EDTA	Disodium salt of ethylenediamine tetraacetic acid
EtOH	Ethanol
FPA	Free phenolic acids
GC-MS	Gas Chromatography- Mass Spectrometry
HPLC	High Performance Liquid Chromatography
MeOH	Methanol
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
PrOH	Propanol
TCA	Trichloracetic acid
TBA	Thiobarbituric acid
TFA	Trifluoroacetic acid
TMS	Trimethylsylil group

I. Introduction

Various medicinal properties have been ascribed to natural herbs. Medicinal plants constitute one of the sources of new pharmaceuticals and healthcare products. A whole range of plant-derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating disease are now being described as functional foods, nutriceuticals and nutraceuticals. Plant-derived products are also increasingly accepted and used in the cosmetic industry. Or, singlet oxygen quenchers, and metal chelators (Rice-Evans et al., 1997).

The widespread use of traditional herbs and medicinal plants has been traced to the occurrence of natural products with medicinal properties. The roles of herbal tea in disease prevention and cure have been attributed, in part, to antioxidant properties of their constituents-liposoluble vitamins A and E, the water soluble vitamin C, and a wide range of amphipathic molecules, broadly termed phenolic compounds. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers, and metal chelators (Rice-Evans et al., 1997 and Morel et al., 1994).

The importance of antioxidants in the maintenance of health and protection from the damage induced by oxidative stress (implicated in the risk of chronic diseases), is coming to the forefront of dietary recommendations, the development of functional foods and the extraction of novel potentially therapeutic compounds from medicinal plant. Fruit, vegetables, beverages and grains are rich in the polyphenolic family of antioxidant phytochemicals, the flavonoids. Flavonoids represent the single, most widely occurring group of phenolic phytochemicals (Rice-Evans et *al*., 2001). Among flavonoids, phenolic acids, tannins, and tocopherols are pointed out as the most common natural source of anti-oxidant phenolics (Angelo et al., 2007).

The aim of this work was to test five moss species for their antioxidant and free radical scavenging activity. HPLC and GC- MS have become the analytical methods of choice for identification of compounds responsible for their activity.

II. Theoretical part

1. Introduction to polyphenolic compounds

Phenolic phytochemicals are important aromatic secondary metabolites in plants, many of which are commonly substituted by sugar moieties such as glucose, arabinose, xylose, rhamnose and galactose. Significant amounts of phenolic compounds frequently occur in foods such as fruits and vegetables and are routinely consumed in our diet. They importantly attribute to the sensory qualities (colour, flavour, taste) of fresh fruits, vegetables and their products. In addition, many phenolic phytochemicals have antioxidative, anticarcinogenic, antimicrobial, antiallergic, antimutagenic and antiinflammatory activities (Kim et al., 2000).

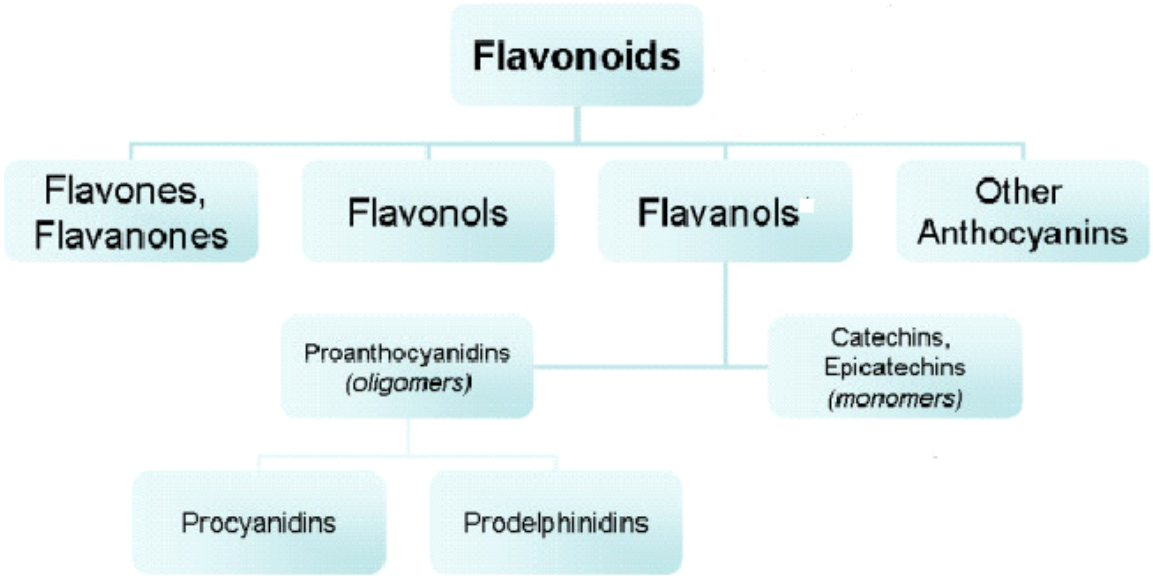
Some phytochemicals, including flavonoids in fruits and vegetables, consumed as part of our daily diet, may reduce the risk of cardiovascular disease (Cook & Samman, 1996). Epidemiological studies show a significant inverse relationship between dietary intake of fruits and vegetables and the risk of coronary heart disease (Knekt & Maatela, 1996). The distribution and composition of phenolic phytochemicals are affected by maturity, geographic origin, growing season and processing procedures.

The measurement of antioxidant activity of individual compounds may lead to a misleading conclusion due to frequently observed antagonistic or synergistic interactions of various components of foods. (Vinson et al, 2001).

Flavonoids are hydrogen-donating radical scavengers (antioxidants). By complexing iron ions, flavonoids suppress the superoxide-driven Fenton reaction (Rice-Evans et al., 1996). Copper complexation is also an important activity of certain flavonoids, espetially those with the catechol structure in B-ring (Brown et al., 1998)

By reducing the α-tocoferoxyl radical flavonoids regenerate α-tocoferol and also quench singlet oxygen (Rice.Evans et al.; 1996).

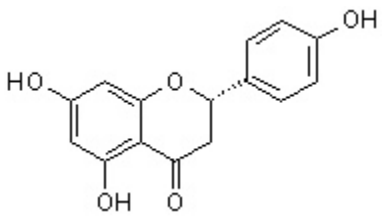
1.1. Classification of flavonoids



Anthocyanins

Anthocyanins are acylglycosides and glycosides of anthocyanidines. They are usually C_3 monosides, biosides, and triosides although there are also 3, 5- and 3, 7-diglycosides (Strack and Wray, 1994).

Flavanols (Catechins).



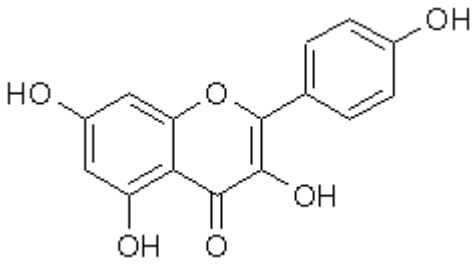
catechin

Catechins are found mainly in brewed tea (Bronner and Bleecher, 1998) and in red wine (Goldberg et al., 1998).

Flavanones

Flavanones are predominantly in citrus, where they are usually found as mono- and diglycosides.

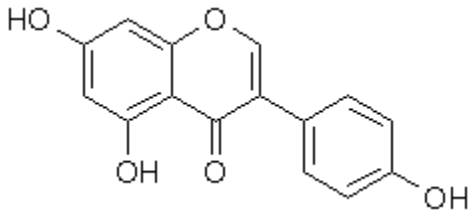
Flavones and Flavonols



flavonol

Flavones and flavonols are usually found in plants as O-glycoside. The flavonols have a hydroxyl group at $C_{3,}$ where the flavones have hydrogen.
The vegetables, herbs, and teas containing flavones, flavonols, and flavon glycosides.

Isoflavones



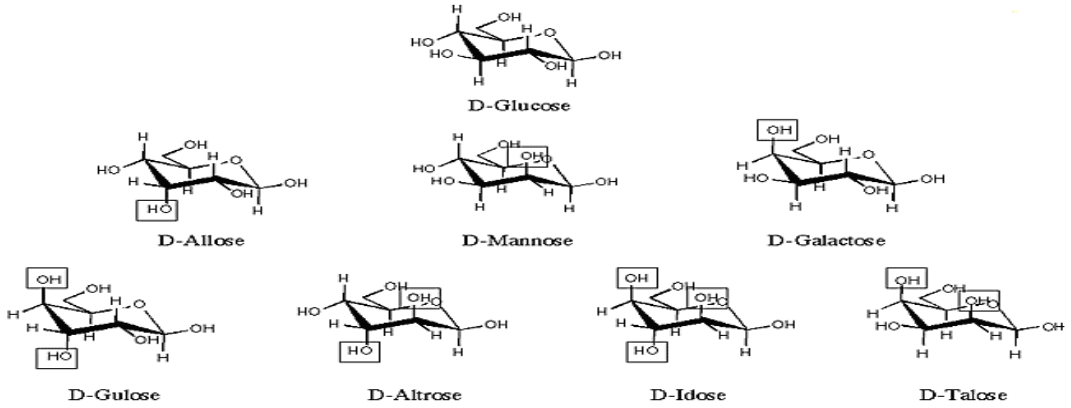
isoflavone

About 20 of the 1300 species of legumes are eaten by people. Soy and its products are the most widely studied for their isoflavone content (Mazur et al., 1998). At least 15

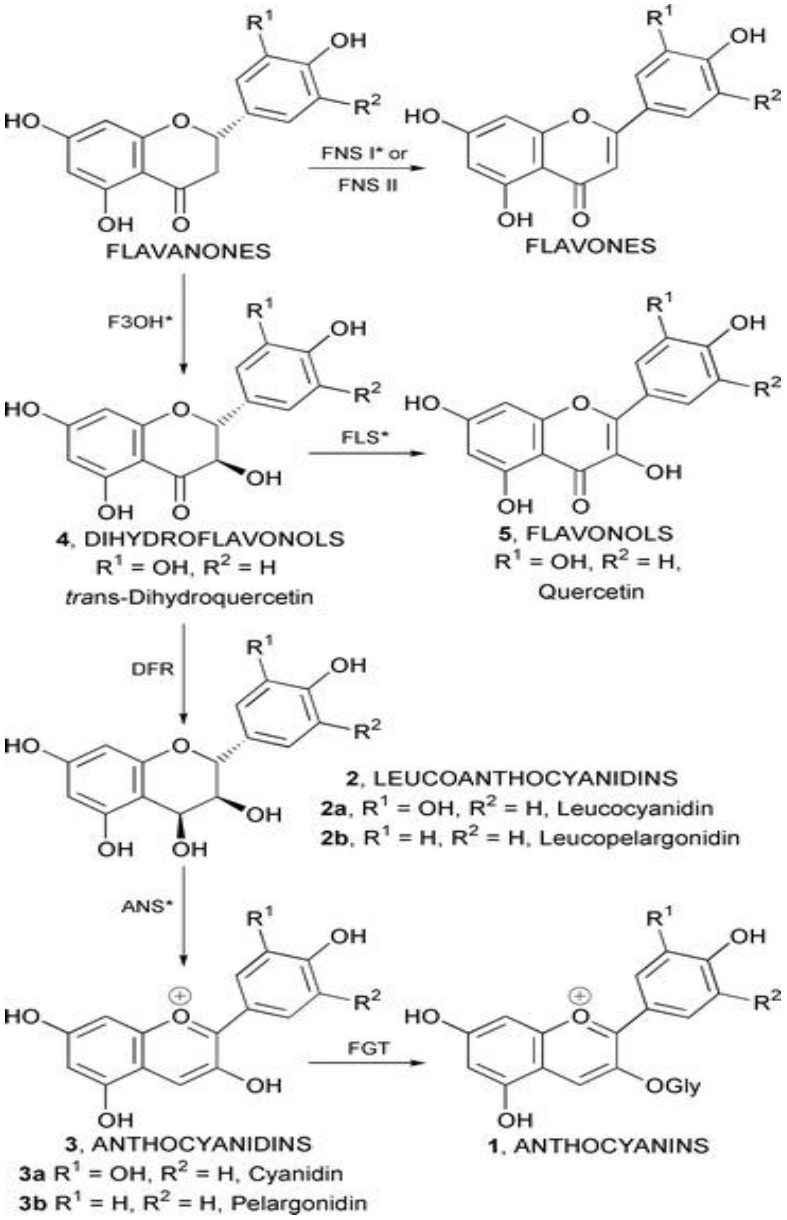
isoflavones are found in food, usually as glycosides, although aglycons are found in fermented soy products. Low levels of isoflavone are found in other legumes (Bingham et al., 1998).

Very often flavonoids exist in form of glycosides,that means they have a sugar moiety. Here are some examples of typical sugars found in studied mosses.

Sugar	**Rt(min)**	**moss sample**
allose	9.998	*M.marginatum*
erythritol	6.992	*M.marginatum*
D-fructose	9.123	*M.marginatum*
D-galactose	10.025	*L.glaucum*
galactofuranose	9.582	*L.glaucum, M.marginatum*
glucitol	10.456	*L.glaucum*
glucose	11.01	*L.glaucum, M.marginatum*
mannitol	11.819	*M.marginatum*
mannose	11.437	*M.marginatum, L.glaucum*
mannopyranose	10.039	*L.glaucum, M.marginatum*



1.2. Biosynthesis of flavonoids



www.rsc.org

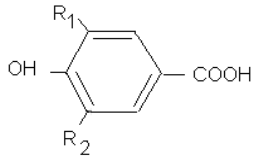
1.3. Phenolic acids

Phenolic acids are a group of natural products commonly found in many cereal grains, fruits, plants and herbs. They may vary in structure due to difference in number and position of the hydroxyl groups on the aromatic ring. As a group, these naturally occurring compounds have been found to be strong antioxidants against free radicals and other reactive oxygen species (ROS), the major cause of many chronic human diseases such as cancer and cardiovascular diseases (Kim et al., 2005).

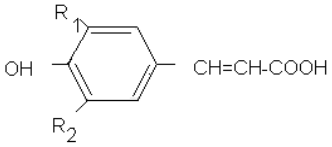
Analytical procedures can significantly affect the antioxidant activity of phenolic acids because of the variable contents and types of phenolic acids through different sample preparations (Lehtinen & Laakso, 1997), extraction and hydrolysis procedures. The hydrolysis method, in particular, can affect the yield and profile of phenolic acids if they exist in form of esters. The ester bond is break down during the hydrolysis

1.3.1. Phenolic acids derivatives

Benzoic acid derivatives



Cinnamic acid derivatives



	R_1	R_2	
p-Hydroxybenzoic acid	H	H	p- Coumaric acid
Protocatechiuc acid	H	CH	Caffeic acid
Vanillic acid	CH3O	H	Ferulic acid
Syringic acid	CH3O	CH3O	Sinapic acid

Other structures are showed in Attachment no. 3.

1.3.2. Mechanism of action of polyphenolic compounds

The antioxidant capacity of phenols is generally ascribed to the reaction with oxidants to form resonance-stabilized phenoxyl radicals (Baum and Perun, 1962). This activity is strengthened by the presence of a second hydroxyl group, as in caffeic and protocatechuic acids, through the formation of an intramolecular hydrogen bond. Moreover, in the case of copper-catalyzed oxidation, only the presence of the two hydroxy groups in the ortho position (caffeic and protocatechuic acid) produced the formation of the Cu(II)-phenolic acid complex, evidenced by the shift of their spectra, resulting in a chelating effect of copper, as already described for caffeic acid (Nardini et al., 1995).

The greatest antioxidant capacity of hydroxycinnamic acid derivatives is linked to the presence of the propenoic side chain, instead of the carboxylic group of benzoic acid derivatives; the conjugated double bond in the side chain could have a stabilizing effect by resonance on the phenoxyl radical, thus enhancing the antioxidant activity of the aromatic ring. Remarkably, hydroxycinnamic acid derivatives are the most widely represented phenolic acids in food vegetables, strengthening their potential role as nutritional antioxidants. (Natella, 1999)

Unlike hydroxycinnamates, hydroxybenzoic acid derivatives are mainly present in the form of glucosides in foods. The most common forms are *p*-hydroxybenzoic, vanillic, and protocatechuic acids. Ellagic acid is a dilactone of hexahydroxydiphenic acid, which in turn is a dimeric condensation product of gallic acid. (Mattila, 2002)

Extracts of various medicinal plants containing flavonoids have been reported to possess antimicrobial activity (Colombo; Li and Tereschuk). The antibacterial activities of isoflavonoids and flavonoids and glycosides of luteolin and apigenin have been reported (Gnanamanichan and Miski). In this respect, the most investigated taxa are the angiosperms while few data are currently available about other groups of plants, including bryophytes (Asakawa and Markham).

2. Mosses

2.1. Mosses in plant Kingdom

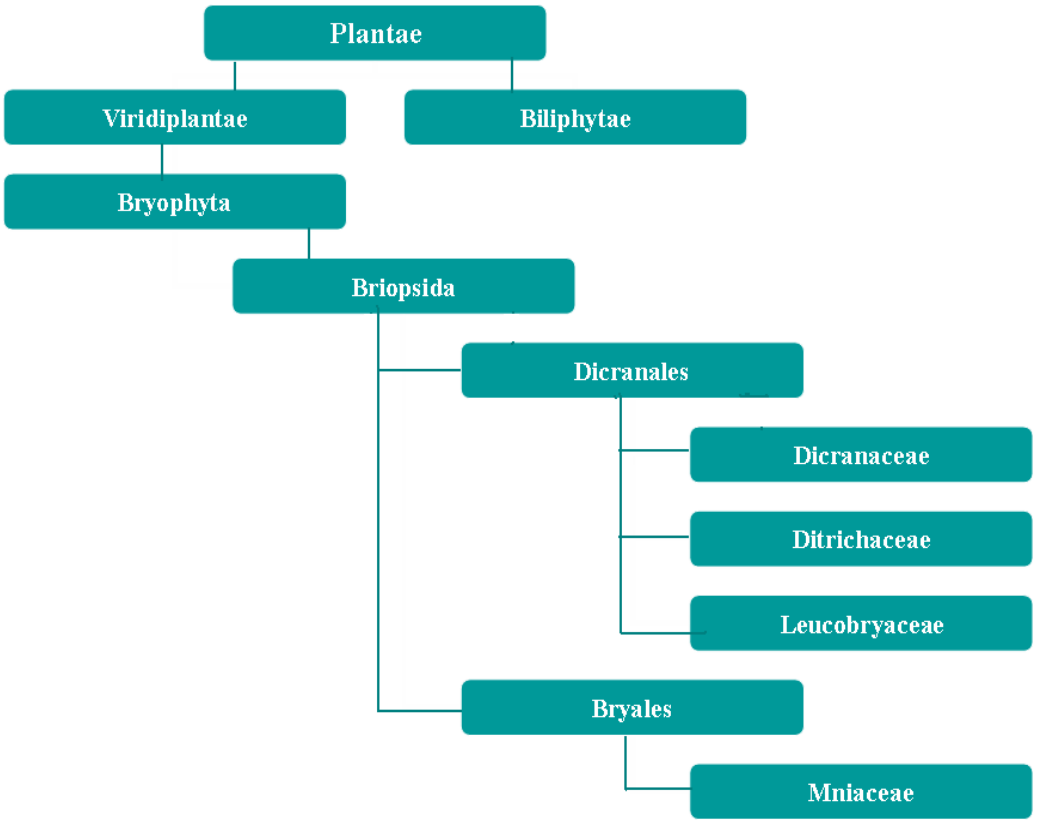
The bryophytes comprise more than 20,000 species world-wide. They are divided into three classes, Musci (mosses 14000 species), Hepaticae (liverworts 6000 species) and Anthocerotae (hornworts 300 species). Some bryophytes emit intense sweet-mushroomy, sweet-woody, turpentine, fungal-like or seaweed-like and carrot-like scents (Asakawa, 2004).

In Bryophytes, which are the simplest land plants, anatomical barriers are less effective and, as a consequence, the synthesis of particular molecules, secondary metabolites with antimicrobial activity: the so-called 'chemical barrier' (Harborne, 1988), is the most effective defense mechanism. Defense substances belong to a wide range of different chemical classes including flavonoids and isoflavonoids (Smith, 1996). Bioflavonoids in mosses are also reported as possible chemical barriers against micro-organisms (L and Geiger).

2.2. Mosses of orders *Bryales* and *Dicranales*

Gametophytes of five moss species occurring commonly in central Europe: *Ceratodon purpureus* (Ditrichaceae), *Dicranum polysetum* (Dicranaceae), *Leucobryum glaucum* (Leucobryaceae), *Mnium marginatum* (Mniaceae), were collected in South and East regions of the Czech Republic in spring 2005. The samples were identified by Dr. Vladimír Chobot, PhD.

2.3. Taxonomy of mosses



Previously isolated class of constituents

Moss	Previously isolated constituents	Yields of extract (w/w %)
C.purpureus	Lipids,unstat. f.a, flavonoids	1.83
D.polysetum	Lipids, acetylenic acids	5.37
D.scoparium	Lipids,acetylenic ac.,flavonoids	6.67
L.glaucum	Waxes, sterols	2.56
M.marginatum	Terpenes	4.15

2.4. Previously studied phenolic compounds in mosses

The antibiotically active substances of *Atrichum, Dicranum, Mnium, Polytrichum,* and *Sphagnum* spp. are considered to be polyphenolic compounds (McCleary & Walkington, 1966). In particular, flavonoids, including phenolic acids, are the main group of phenols from mosses and many new compounds have been detected in the last few years (López-Sáez et al., 1996). Flavones from bryophytes can be subdivided into derivatives of apigenin, luteolin, scutellarein, isoscutellarein, hypolaetin and tricetin (Huneck, 1983). Among the monoflavonoids apigenin, luteolin, kaempferol and orobol derivatives are the usual ones found in mosses (Zinsmeister; Markham). Biflavonoids from apigenin, luteolin and eryodictiol are also an important source of secondary metabolites from mosses (Geiger; Geiger; Markham; López, 1994).

3. Antioxidant and free radical scavenging activity

3.1. Definition of antioxidant activity

Reactive free radicals, such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and peroxyl radical ($ROO^{\bullet}$), are particularly reactive and are known to be a biological product in reducing molecular oxygen (Williams & Jeffrey, 2000). Damage mediated by free radicals results in the disruption of membrane fluidity, protein denaturation, lipid peroxidation, oxidative DNA and alteration of platelet functions (Fridovich, 1978 and Kinsella et al., 1993), which have generally been considered to be linked with many chronic health problems such as cancers, inflammation, aging and atherosclerosis.

An antioxidant, which can quench reactive free radicals, can prevent the oxidation of other molecules and may, therefore, have health-promoting effects in the prevention of degenerative diseases (Shahidi, 1997). The interest in antioxidants has been increasing because of their high capacity in scavenging free radicals related to various diseases (Silva, Souza, Rogez, Rees, & Larondelle, 2007). In this respect, phytochemicals from

fruits have been shown to possess significant antioxidant capacities that may be associated with lower incidence and lower mortality rates of degenerative diseases in human (Javanmardi et al., 2003). The antioxidant properties of fruits vary depending on their content of phenolic components and vitamins C and E, carotenoids, flavonoids (Saura-Calixto & Goni, 2006).

3.2. Studied activity of mosses

Total phenolic content was determined using Folin-Ciocalteau reagent. Reducing power, scavenging 1, 1-diphenyl-2-picrylhydrazyl and nitric oxide radicals and inhibition of site-specific and nonsite-specific hydroxyl radical-mediated 2-deody-D-ribose degradation. Caffeic acid was used as positive control for free radical scavenging and antioxidant activity.

4. Analytical methods

Two moss species were analyzed. Ethanolic extracts of *Lucobryum glaucum* and *Mnium marginatum.*

4.1. HPLC analysis of phenolic compounds

High performance liquid chromatography has many applications including separation, identification, purification, and quantification of various compounds. HPLC offers a number of advantages over other techniques. A wide range of column packing materials is available for specific applications and the columns can be used very many times. Analysis time can be relatively short, retention times of compounds under set conditions are reproducible and the nature of equipment allows a high degree of automatisation (Beerman et al, 2003).

4.1.1. Applications for HPLC

Preparative HPLC refers to the process of isolation and purification of compounds. Important is the degree of solute purity and the throughput, which is the amount of compound produced per unit time. The information that can be obtained includes identification, quantification, and resolution of a compound. (www.pharm.uky.com)
Analytical HPLC focuses to obtain information about the sample compound.
Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a known solution. It involves injecting a series of known concentrations of the standard compound solution onto the HPLC for detection. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected. (www.pharm.uky.com).

4.1.2. Column

The column is the most important part of the whole system, where the separation takes place. The design and construction of columns is still developing. Typical columns are constructed of high quality stainless steel and are highly polished to minimize the effects of the wall on peak broadening. Pre-columns in front of the main column may prolong column life by filtering the solvent and trapping microimpurities. The partial substitute for pre-column is a filter, consisting of stainless steel porous firt inserted between the valve and the column. It is of a serious importance that the pressure conditions should be monitored during the analytical process. A sudden pressure rise can be very harmful to the column. Other causes of pressure fluctuation are air bubbles or pump malfunctions. (Homan and Anderson, 1998, Seppänen-Laakso et al. 2001).

4.1.3. Solvent system

The choice of a solvent for use in mobile phase is dependent on the nature of compounds to be analyzed. The polarity of solvent system is one of the first factors to consider,

especially in case of polar phenolic acids and less polar flavonoids. In formulating mixed solvents, it is essential that they be fully miscible with each other. For reproducible results when mixing solvents to prepare an eluent, it is important to measure out each of the solvents separately before mixing (and do not use a single measuring cylinder and make up each solvent to appropriate mark).
All solvents contain dissolved air, the solubility of which increased at high pressure. With the sudden release of pressure at the end of chromatographic column, bubbles can form that cause pressure fluctuations and interfere with detection. The simplest and least hazardous method of removing air from solvents is to purge them with helium gas, or to use an ultrasonic bath before starting the HPLC analysis (Christie, 1987).

4.1.4. Pump

One of the primary requirements for an HPLC system is the pump, capable of propelling the mobile phase through the microparticulate stationary phase in a column under high pressure. Pumps must be manufactured from materials resistant to any of the mobile phase. The pump should have a low internal volume and it must be capable of delivering solvents of set value, so that any variations in retention times are not significant. (Christie, 1987).

4.1.5. Standards

As standards were used phenolic acids and flavonoids, both aglycons and glycosides.
All solvents were of analytical grate and were degasses using ultrasonic bath.
The list of used standard is in Practical part.

4.2. GC-MS analysis of phenolic compounds

4.2.1. Introduction to GC-MS

For qualitative analysis, GC-MS is a technique where one experiment can generate a wide range of information. The GC performs separation and MS masters in separated component identification. The Gas Chromatography-Mass Spectrometry is a good analytical method to detect and separate phenolic acids and flavonoid aglycones. When these molecules pass through the column, they are held inside according to their polarity and their molecular mass.

To analyze these products, GC-MS requires a derivatization procedure first.

Derivatisation was used in order to make these molecules less polar, more volatile and more thermally stable so as to make them go out earlier (the column is polar) and have a stable gas state (more soluble into gas carrier).

Reverse phase column was used.

MSTFA reagent was used for derivatisation. It´s a nucleophilic substitution. The TMS (trimethylsylil group; weight: 72) from MSTFA attaches hydroxyls groups (OH) and carboxylic groups (COOH) of molecules. So, according to the number of TMS groups attached, we can calculate the weight of derivatives.

5. Hydrolytic cleavage

Hydrolysis was performed to remove the sugar moiety of flavonoids to obtain aglycone, which was later on analyzed by GC-MS and HPLC.

Ethanolic extracts of two moss species were hydrolyzed and analyzed. Extract of *L. glaucum* and *M. marginatum*

5.1. Extractable phenolic acids

In this procedure we followed the materials of Kim at al., 2005, using two different types of hydrolysis – acidic and basic one. As first was extracted acidified solution of mosses with ethyl ether. Ether layer contained free phenolic acids (FPA). The polar part was hydrolyzed under basic conditions. The ethyl layer fraction contained alkaline – hydrolysable phenolic acids (AHPA). The polar phase was again hydrolyzed, this time was performed acidic hydrolysis. Even this fraction was partitioned with ethyl ether and this layer contained acidic-hydrolysable phenolic acids.
All three fractions – FPA, BHPA, AHPA, were analyzed by GC- MS, HPLC- PDA

5.2. Thiolysis

Thiolysis is complete hydrolytic cleavage in presence of benzyl mercaptan.
Thiolysis is used in case of presence of proanthocyanidins, known as condensed tannins, widely distributed in the plant kingdom and they represent a ubiquitous group of plant phenolics (Weinges et al, 1968). Tannins are complex polyphenolic metabolites of plants based upon two principal structural themes – oligomeric flavan-3-ols (proanthocyanidins) and poly-3,4,5-trihydroxyaroyl esters (gallotannins and ellagitannins) (Haslam, 2007).
During this procedure the ester bond is break down and results into two intermediate products, monomeric and oligomeric subunits. Flavan-3-ol derivatives. **(**de Freitas et al, 1998).
In this method was followed work of U. Svedström, with modifications.

5.2.1. Working conditions

While carry out the thiolysis, one has to be very careful while handing with benzyl mercaptan. Whole procedure has to be performed in effective chamber, after each manipulation with mercaptan change gloves and keep them in the chamber. Even a negligible amount can produce very unpleasant smell.

5.3. Acidic hydrolysis

For the determination of individual flavonoid glycosides in plant materials, the glycosides were hydrolyzed and the resulting aglycons were identified and quantified. However, the hydrolysis conditions which result in optimal breakdown of glycosides are too harsh for some of the other phenolic compounds present in the same plant material.

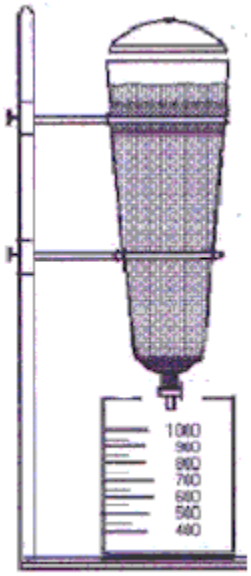
III. Experimental part

1. Plant material

1.1. Preparation of moss extracts

Dried moss was cut using blender, sieved and moistened with small portion of ethanol (96%). The moistened drug was allowed to stand for a period of four hours to allow the drug to imbibe the menstruum and thereby swell to its maximum capacity. The container used should be large enough to accommodate the expansion of the drug. Bottle was covered and protected from light. Drug mixture was transferred into percolator filled with ethanol till the top and again covered with aluminum foil. After one day, the stop cock on the bottom of the percolator was opened and the menstruum was poured in portions and allows percolating through the packed drug. The menstruum dripped through the drug in speed 1 drop/min. Percolate was collected immersed into a flask and evaporated using vacuum evaporator. Flask was weighted first.

Ethanolic extract was obtained by percolation according to Czech Pharmacopoeia 97. Percolation is a method of extraction achieved by the downward displacement of soluble extractive by a suitable solvent through a suitably comminuted drug plant. The process is a combination of maceration and percolation.



Percolator

2. Solvents and reagents

The following chemicals were used in the HPLC and GC-MS analysis and hydrolytic cleavage:

Solvent or reagent	Origin
Chloroform	Merck, Germany
Diethyl ether	Fluka, Chemica, Germany
Ethanol	Altia, Finland
Hexane	Rathburn Chem., UK
Methanol	Rathburn Chem., UK
Petroleum ether	Rathburn Chem., UK
Acetic acid 99,8%	Riedel-de Haen, Germany
Trifluoroacetic acid	Fluka, Chemica, Germany
Benzyl mercaptan 99%	Aldrich, Germany

Preparation of buffer

Buffer was used in mixture with alcohol as solvent in antioxidant assays.

Phosphate buffer solution, pH 7.4.

Potassium Phosphate Monobasic	1%
Sodium Phosphate, Dibasic	1%
Water	98%

Pure substances

Standard	Company	Amount mg/ml	Inj.vol. (µl) for HPLC
apigenin	Extrasynthése, Genay, France	0.2	10
apigenin-7-O-glucoside	Extrasynthése, Genay, France	0.2	20
benzoic acid	Extrasynthése, Genay, France	0.15	30
hyperoside	Extrasynthése, Genay, France	0.2	20
caffeic acid	Sigma	0.2	20
±catechin	Sigma	0.2	20
chlorogenic acid	Fluka,AG Buchs	0.2	20
coumarin	Sigma	0.15	20
4-hydroxycoumarin	Sigma	0.15	10
m-coumaric acid	Fluka, AG Buchs	0.2	30
o-coumaric acid	Sigma	0.1	20
p-coumaric acid	Sigma	0.1	20
2,4-dihydroxybenzoic acid	Fluka, AG Buchs	0.1	20
3,4-dihydroxybenzoic acid	Fluka, AG Buchs	0.1	20
4-hydroxybenzoic acid	Extrasynthése, Genay, France	0.2	10
luteolin	Extrasynthése, Genay, France	0.2	20
luteolin-7-O-glucoside	Extrasynthése, Genay, France	0.2	20
ferulic acid	Sigma	0.15	20
gallic acid	Sigma	0.1	20
protocatechuic acid	Roth	0.2	20
quercetin	Extrasynthése, Genay, France	0.2	10
shikimic acid	Sigma	0.2	10
syringic acid	Sigma	0.25	30
trans-cinnamic acid	Extrasynthése, Genay, France	0.3	10
vanillic acid	Fluka, AG Buchs	0.2	30
vitexin-glucoside	Extrasynthése, Genay, France	0.1	20
vitexin	Extrasynthése, Genay, France	0.1	20

3. Instrumental equipment

3.1. GC-MS

The GC-MS analyses were performed on a Hewlett-Packard (HP) 5890 GC coupled to an HP 5970 quadrupole mass selective detector operated at an ionization voltage of 70 eV with electron impact (EI) mode). Samples were analyzed on an NB-54 fused silica capillary column using an oven temperature from 100 °C to 275 °C at 10 °C/min.

Identification was based on the GC retention times and GC-MS spectra were compared with those obtained from analyses of pure substances during the study, and with those from Division's own library compounds, their methyl esters or trimethylsilyl (TMS) derivatives, and from Wiley 275 L library (John Wiley & Sons, Inc., NJ, USA).

Gas chromatograph	Hewlett Pacard 5890 A	HP, USA
Mass selective detector	Hewlett Pacard 5970 A	HP, USA
Column	NB-54; 15m, i.d.0,2mm thickness: 0,1mm	HNU-Nordion LTD, Finland

3.2. HPLC

Autosampler	Waters 717™
Controller	Waters 600™
Pump	WatersTM 600™
Photodiode Array Detector	Waters 2996™
Column	Hypersil BDS-C18 5 μm, 4,6*150mm

Solvents

Solvent A: 98 % of 0.02 % TFA + 2 % of MeOH
Solvent B: 95 % of MeOH + 5 % of 0.02 % TFA

4. Antioxidant and free radical scavenging activity

4.1. Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteau reagent, prepared according to Czech Pharmacopoeia 4.

The Folin-Ciocalteau reagent is a solution of complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. It oxidises phenolates, reducing the heteropoly acids to a blue Mo-W complex. The phenolates are only present in alkaline solution but the reagent and products are alkali unstable. Hence a moderate alkalinity and a high reagent concentration were used.

Sodium carbonate:
Solution was prepared according to Czech Pharmacopoeia 4:350/ I.
100 g of anhydrous sodium carbonate was dissolved in 1 l, prepared in the time of use.

Solutions of moss extracts:

Solvent: PrOH + water aa = basic solution R
Approximately 5 mg of extract was dissolved in 2 ml of R = Z
Conc. No 1: dilution from Z: 0.8 ml of Z + 1.2 ml of R (c= 1 mg/ml)
Conc. No 2: dilution from 1: 0.6 ml of 1 + 0.6 ml of R (c= 0.5 mg/ml)

Determination

An amount of 0.2 ml of solution was pipetted into cell (cuvette), after which 600 μl of FC reagent was added and waited for 15 min. After this, 600 μl of sodium carbonate was added and mixed on ultrasound bath. The absorbance was measured after 30 min (760 nm). Blind sample: the same procedure, but instead of a sample, solution R was pipetted. The procedure was repeated three times.

Calibration curve of gallic acid

For total phenolic content as gallic acid equivalents in the dried extract.

M_r = 170.12

8.5 mg of gallic acid was weighted and diluted up to 100 ml in R (PrOH:water; 1:1)

c(mol/l)	A 760 nm			c(mg/l)
5.10^{-4}	1.431	1.247	1.380	85.0
$3{,}75.\ 10^{-4}$	1.075	1.115	1.163	63.75
$2{,}5.\ 10^{-4}$	0.744	0.77	0.779	42.5
$1{,}25.\ 10^{-4}$	0.399	0.390	0.411	21.25
$1{,}10.\ 10^{-4}$	0.320	0.321	0.323	17.00
$8{,}75.\ 10^{-4}$	0.290	0.279	0.299	14.87
$7{,}5.\ 10^{-4}$	0.228	0.229	0.230	12.75
$6{,}25.\ 10^{-4}$	0.188	0.191	0.200	10.625
$5.\ 10^{-4}$	0.155	0.153	0.159	8.5
$3{,}7.\ 10^{-5}$	0.111	0.113	0.117	6.375
$2{,}5.\ 10^{-5}$	0.074	0.071	0.079	4.25
$1{,}25.\ 10^{-5}$	0.034	0.033	0.036	2.125
$5.\ 10^{-5}$	0.011	0.015	0.011	0.85
$3{,}7.\ 10^{-6}$	0.007	0.008	0.006	0.637
$2{,}5.\ 10^{-6}$	0.001	0.001	0.004	0.425

4.2. Griess method

Scavenging of nitric oxide radicals.

Chemicals

Sodium nitroprusside (NPR), Mr = 277.9, c = 694.8mg/25ml of buffer 7.4 (Czech Pharmacopoeia 4).
Solution R = PrOH: buffer 7, 4 (1:1)
Mosses: 10 mg/ 5 ml of R
Caffeic ac.: 3.6 mg/ 10 ml R
Griess reagent: 1% sol. of sulfanilamide in 2% phosphoric acid + 0.1% sol. of dihydrochloride of N-(1-naftyl)ethylendiamine in propanol. Prepared fresh before use.

Procedure
Dilution of mosses and standards:
Z = 10 mg of extract in 10 ml of R
Z/10 = 1 ml of Z + 9 ml of R
Z/100= 0.8 ml of Z/10 +7.2 ml of R

0.9 ml of sample + 0.1ml of NPR solution were mixed and incubated at 25 ° C for 60 min on light. After the Griess reagent was added in 10 seconds intervals, allowed to stand for 10 min on the light and the Absorbance was measured at 546 nm.
Note: If temperature is higher than 35 °C precipitation might occur.

As a positive control was used caffeic acid in these concentrations:
Z = 3.6 ml of caffeic acid / 10 ml of R (c= 0.36 mg/ml)
Z/10 = 1 ml of Z + 9 ml of R (c= 0.036 mg/ml)
Z/ 100= 1 ml of Z/10 + 9 ml of R (c= 0.0036 mg/ml)
Z/ 1000 = 1 ml of Z/100 + 9 ml of R (c= 0.00036 mg/ml)

4.3. Reducing power

Antioxidant activity is probably related to reductive activity. To determine whether the ethanolic extracts of mosses can participate on redox reactions, its ability to reduce iron (III) was assessed. The iron (III) reductive capacity of extracts was assessed spectro-photometrically. (Dorman et al, 2003, with modifications).

Note: it is not possible to use solvent as alcohols or other type of solvents that interact with reagent.

Chemicals

1 % K_3 [Fe (CN) $_6$] – water solution
0.1% $FeCl_3.6H_2O$ – water solution
10 % CCl_3 COOH – water solution

Procedure

0.4 ml of extract dissolved in water, combined with 1 ml of buffer 7.0 (or 6.6) and 1 ml of 1% K_3 [Fe (CN) $_6$].
After 30 min of incubation, 1 ml of 10% CCl_3COOH was added and centrifuged for 10 min (20 000 rot/min).
Incubation: on water bath in 50 °C for 30 min.
A 1 ml solution was then taken into the cells mixed with 1 ml of water and 0,2 ml of $FeCl_3$. After 10 min the absorbance was measured at 700 nm.
Blind sample contained water and $FeCl_3$, absorbance: 0, 0020*0,021
Z= 5 mg/ml: 5 mg of extract in 1 ml of water and 1 ml of buffer 7.0.
Other dilution: 5 mg/ml: 0.8 ml of Z + 0.6 ml of buffer 7.0
2 mg/ml: 0.320 ml of Z + 1.080 ml of buffer
1 mg/ml: 0.160 ml of Z + 1.240 ml of buffer
0, 5 ml: 0, 080 ml of Z + 1,320 ml of buffer

As a positive control were used quercetin, caffeic and ascorbic acids.
Concentrations of quercetin and caffeic acid: Z=3 mg/3 ml, Z/10, Z/100, Z/1000

4.4. Scavenging of DPPH

Scavenging of 1, 1-diphenyl-2-picrylhydrazyl radicals (nitrogen centre free radicals). The moss extracts were capable of scavenging $DPPH^{\cdot}$ free radicals, which is synthetic radical and acts as both an oxidizable substrate and as the reaction indicator molecule.

DPPH: 8.1 mg 95% DPPH/ 52 ml of MeOH
Mosses: 10 mg/5 ml MeOH (stock solution – Z)
Caffeic acid: 3.6 mg/ml MeOH (Z)

Procedure

1.4 ml of sample (samples were diluted according to the table) was mixed with 0.1 ml of DPPH and kept in a room temperature. The absorbance was measured after 10 min(517 nm).
Baseline: MeOH
Blind samples: 1.4 ml of sample and 0.1 ml of MeOH – for caffeic acid
1.4 ml of sample without DPPH

4.5. Fenton´s reaction

Inhibition of site-specific and non-sitespecific hydroxyl radical-mediated 2-deoxy-D-ribose degradation.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$$

4.5.1. The nonsite- specific

In the presence of EDTA as chelator which chelates ferric ions. OH radicals are formed in the solution, shows ± scavenging of OH radicals.

Chemicals: 500 μl of extract was dissolved in buffer 7.4

100 μl of 28 mM deoxy-D-ribose dissolved in buffer 7.4.

200 μl of solution of $FeCl_3$ / EDTA

100 μl of 1.0 mM ascorbic acid dissolved in water.

1ml of this mixture was incubated in 37 °C for 60 min. After 50 μl of 2.0% BHT was added, then 1.0ml of 2% CCl_3 COOH and on the end 1 ml of 1.0 % thiobarbituric acid was added. Solution was mixed and incubated for 20 min in the water bath and later on cooled for 5 minutes on ice.

After 2 ml of BuOH (or in BuOH: buffer -15:1) was added, centrifuged for 5 min/3000 rpm, and then the absorbance of organic layer was measured at 523 nm.

4.5.2. The site-specific

Without EDTA (buffer was added instead of it), Fe forms chelate together with the deoxyribose or with added substance. Mosses were capable of scavenging of Fe ions.

Procedure

Whole procedure was the same as in the case of nonsite-specific but instead of solution of $FeCl_3$/ EDTA, 100 mM $FeCl_3$ was added and instead of EDTA, buffer (1:1, v/v) was added.

Alcohols and DMSO can not be used as solvents.

Amount of compounds:

- sol.$FeCl_3$: M=270, 30 ($FeCl_{3.}$ $6H_2$ 0)
- 100 mM $FeCl_3$: 27,03 mg/ml
- Deoxyribose: M = 134, 13, 28 mM deoxyribose : 3.8 mg/ml
- Ascorbic acid: 1.0 mM: 176.13 mg/l = 4, 4 mg/25ml
- EDTA: M= 292. 24…104 μM = 30. 39 mg/l
- 1% thiobarbituric acid: 500 mg/50 ml….2,5 g/25
- H_2O_2: 1.0 mM = 3.4 mg/ml = 1 drop (11, 33)
- BHT: M=220.36, 2%: 200 mg/10 ml of EtOH
- CCl_3 COOH: 2.80 mg/ 100 m
- Mosses: Z = 2.5 mg/ml

5. Hydrolytic cleavage

5.1. Extractable phenolic acids

An aliquot of the ethanolic extract of each sample was re-dissolved in 1 ml of acidified water (pH 2 with HCl) and partitioned with 1 ml of ethyl ether, three times. The combined ethyl ether layer was evaporated to dryness and re-dissolved in MeOH. This layer contained FPA – free phenolic acids.

The water phase was neutralized to pH 7 with 2 M NaOH and dried using an oven at 100°C. The residue was dissolved in 1 ml of NaOH and the acidic hydrolysis was performed, water bath at 80 °C for 2h. The solution was then acidified to pH 2 and extracted with ethyl ether as mentioned above. Ether layer was dried and residue was dissolved in MeOH. This phase contained BHPA – alkaline hydrolysable phenolic acids.

The remaining water phase was treated with 1 ml of 6 M HCl and acidic hydrolysis was performed for 45 min at 80°C on water bath. The solution was again partitioned with ethyl ether and ether layer was dried and residue dissolved in MeOH. This phase contained AHPA –acid- hydrolysable phenolic acids (Kim at al., 2005).

All three fractions – FPA, BHPA, AHPA, were analyzed by using GC-MS, HPLC-PDA.

5.2. Thiolysis

5.2.1. Pycnogenol and Crataegus laevigata

0.5 g of powdered leaves and flowers of *Crataegus* (or pycnogenol as second standard) was extracted with a mixture of MeOH and water (7:3), first with 20 ml then with 15 ml, in a ultrasonic bath for 15 min. It was then extracted with 10 ml of MeOH also in ultrasonic bath for 15 min and the sediment was finally washed with 5 ml of MeOH. The solution was filtered through cotton fabric and extracted to eliminate chlorophyll and lipophilic compounds with petroleum ether (3 times 10 ml) that was rejected. The extract was evaporated to dryness and dissolved in 1 ml of MeOH-water (1:1).

Reaction mixture contained: 100 μl of crataegus solution (or pycnogenol), 50 μl of benzyl mercaptan 5% (v/v) in EtOH (0.2ml) and 50 μl of acetic acid. The reaction mixture was heated for 60 min at a temperature of 95 °C.
Reaction product was evaporated (during the weekend in chamber), dissolved in 100 μl of EtOH and analyzed. (Svedström, 2000).

5.2.2. Moss extracts

100 μl of solution of moss extract was mixed together with 50 μl of 5% benzyl mercaptan (v/v) in EtOH (0.2 ml) and 50 μl of acetic acid. The reaction mixture was heated for 60 minutes at 95 °C. Reaction product was evaporated (during the weekend in a chamber), dissolved in 100 μl of EtOH and analyzed by HPLC-PDA and GC-MS.

5.3. Acidic hydrolysis

5.3.1. Pure substances

Pure substances: kaemferol-3-glucoside, hyperoside, naringenin-7-O-glucoside.
Reaction mixture contained: 0.1 mg of these compounds was dissolved in 1 ml of mixture of 1.2 M HCl and MeOH (1:1) and hydrolyzed for 2h at 80 °C. After the

solutions were evaporated using oven set at 100 °C, the samples were analysed by HPLC. For GC-MS analyses the samples were also derivatized with MSTFA.

5.3.2. Moss extracts

Mnium marginatum

32 mg of the original ethanolic extract was dissolved in 2 ml of ethanol, 200 μl of water and extracted with hexane. 100 μl of the polar phase was mixed with 800μl of hydrolytic mixture and hydrolyzed for 2 h on the water bath at 80 °C.

Leucobryum glaucum :

25 mg of original ethanolic extract was dissolved in 1ml of EtOH, sonicated and mixed with hexane. 100 μl of water was added. 200μl of the polar phase was mixed with 1 ml the mixture of 1.2 M HCl and MeOH (1:1) and hydrolyzed for 2 h on a water bath (80°C).

6. HPLC analysis

6.1. Sample preparation

Samples of mosses and of pure substances were dissolved in MeOH and injected. In case of mosses, samples were first defatted using hexane and filtered and polar phase was used for further analysis.

6.2. Run conditions

Solvents were prepared separately and sonicated in ultrasonic bath for 15 min to remove air bubbles.

Solvent system: A: 98% of 0, 02% TFA + 2% of MeOH

B: 95% of MeOH + 5% of 0. 02% TFA

UV range: 200-500 nm

Normal pressure range: 1500-2000 psi.

HPLC analyses were done using the following**,** linear gradient elution system on a reverse-phase HPLC column

	time (mins)	flow	A%	B%	curve
1	0	1	95	5	6
2	50	1	5	95	6
3	60	1	5	95	6
4	65	1	95	5	6
5	75	1	95	5	6

7. GC-MC analysis

7.1. Sample preparation

The samples were first evaporated to dryness and then derivatized (silylated) with MSTFA. After incubation for 20 minutes at 120 °C, a 3 µl sample was injected.

7.2. Run conditions

Starting oven temperature was 100 °C which increased 10 °C/min up to 275 °C. Detector and injector temperature was set at 275 °C.

IV. Results

1. Antioxidant and free scavenging activity

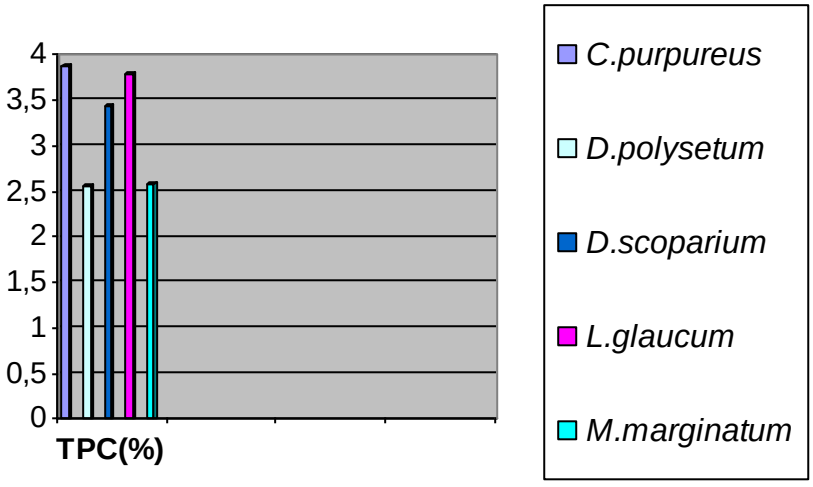
Original numeric results are in attachment no. 4. All presented values were calculated from the experimental repetitions at least and reported with ± 95 %.

1.1. Total phenolic content

Total phenolic content = percentage of gallic acid equivalents in the dried extract.

Mosses

moss	Z (c=2.5mg/ml)	1 (c=1mg/ml)	2 (c=0.5mg/ml)
D. polysetum	0.883	0.465	0.236
	0.968	0.386	0.239
	0.838	0.467	0.234
C. purpureus	1.122	0.778	0.388
	1.202	0.743	0.350
	1.133	0.742	0.373
D. scoparium	0.905	0.700	0.334
	1.342	0.679	0.326
	1.336	0.714	0.319
L. glaucum	1.142	0.706	0.350
	1.425	0.676	0.355
	1.438	0.700	0.355
M. marginatum	1.271	0.517	0.237
	1.253	0.514	0.247
	0.725	0.517	0.233



1.2. Griess method

Dilution of mosses and standards:

Z = 10 mg of extract in 10 ml of R

Z/10 = 1 ml of Z + 9 ml of R

Z/100= 0.8 ml of Z/10 +7.2 ml of R

R: PrOH: water (1:1)

Dilution of Z, Z/10:

Sol.of extract (ml)	R (ml)
0.9	0.0
0.8	0.1
0.7	0.2
0.6	0.3
0.4	0.4
0.3	0.6
0.2	0.7
0.1	0.8

Dilution of Z/100:

Z/10 (ml)	R (ml)
0.9	0.0
0.8	0.1
0.7	0.2
0.6	0.3
0.5	0.4

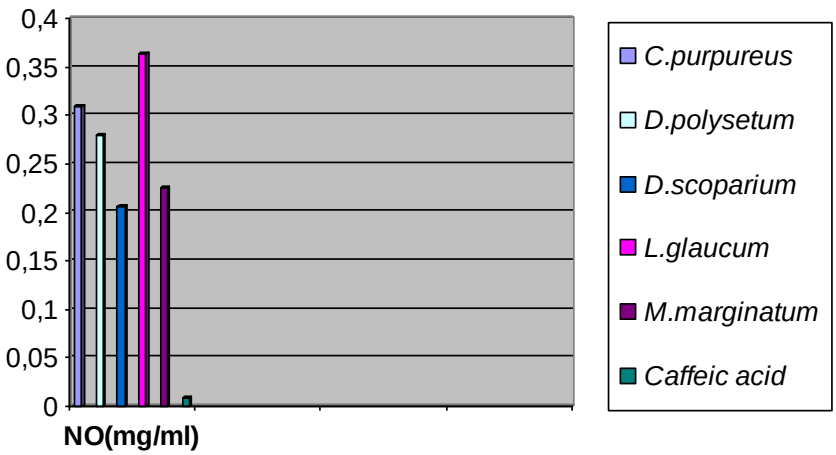
As a positive control was used caffeic acid in these concentrations:

Z = c: 0.36 mg/ml, dissolved in R

Z/10 = 1 ml of Z + 9 ml of R (c= 0.036mg/ml)

Z/ 100= 1 ml of Z/10 + 9 ml of R (c= 0.0036 mg/ml)

Z/ 1000 = 1 ml of Z/100 + 9 ml of R (c= 0.00036 mg/m



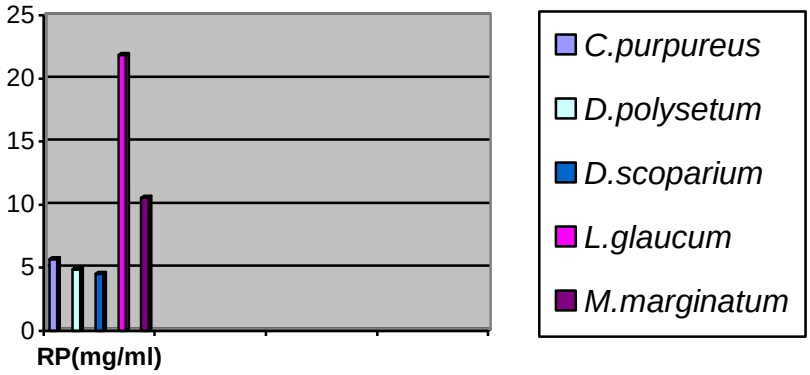
Experiment was repeated three times.

1.3. Reducing power

As a positive control were used quercetin and caffeic acid.

Concentrations of quercetin and caffeic acid: Z=3 mg/3 ml

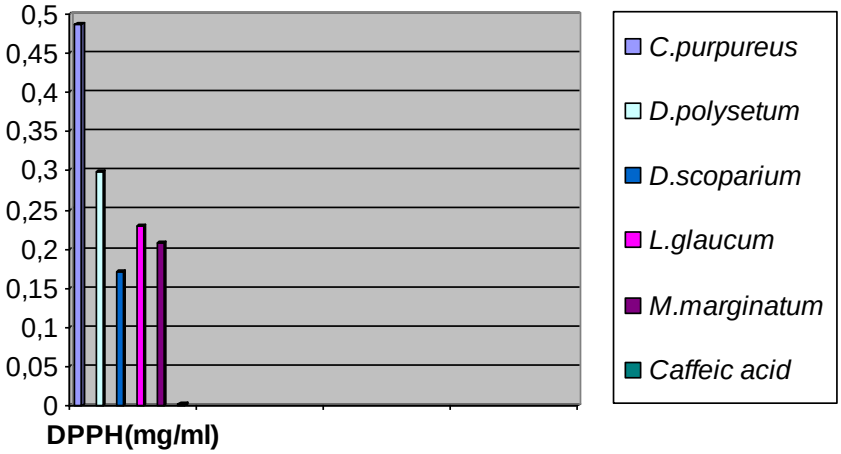
Z/10, Z/100, Z/1000.



RP = reducing power (mg of ascorbic acid equivalents per g of the dried extract).

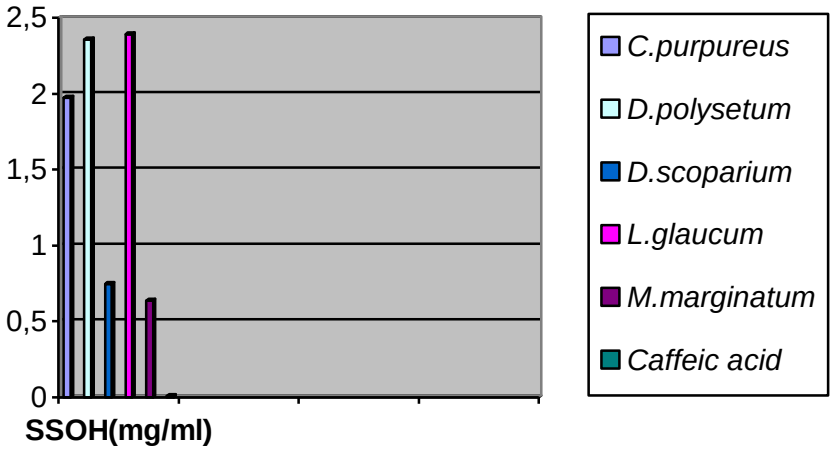
1.4. Scavenging of DPPH$^{\cdot}$

DPPH = 1, 1-diphenyl-2-picrylhydrazyl (EC_{50} mg/ml).



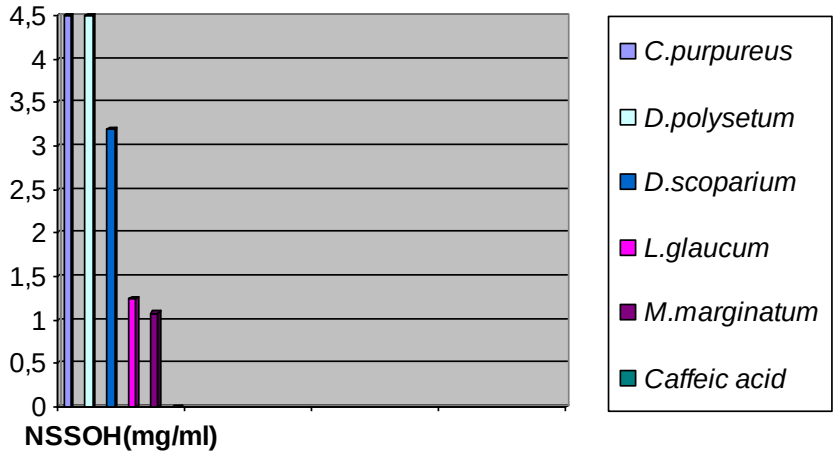
1.5. Fenton´s reaction

1.5.1. Site-specific



Site-specific hydroxyl radical-mediated 2-deoxy-D-ribose degradation (EC_{50} mg/ml).

1.5.2. Nonsite-specific

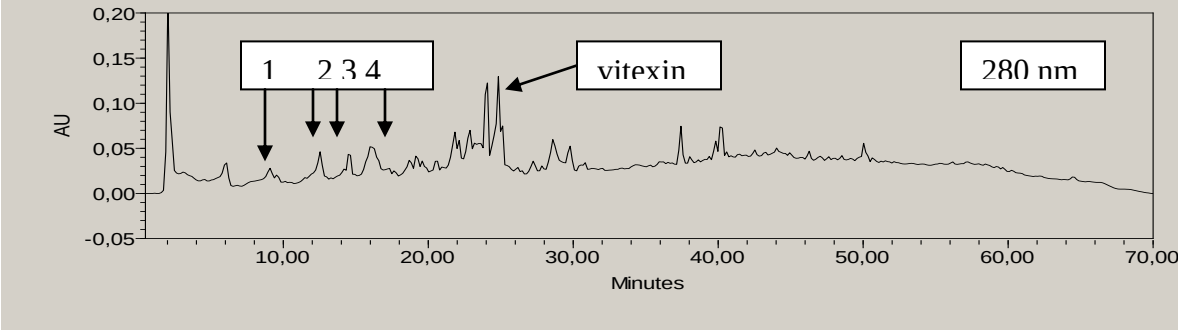


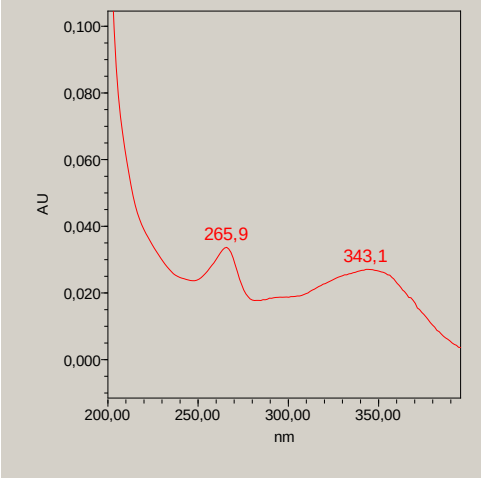
Nonsite-specific hydrolxyl mediated 2-deoxy-D-ribose degradation (EC_{50} mg/ml).

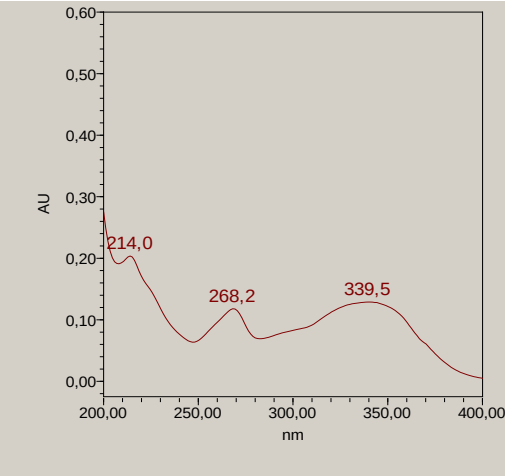
2. HPLC analysis

2.1. HPLC analysis of *Mnium marginatum*

2.1.1. Nonhydrolyzed sample





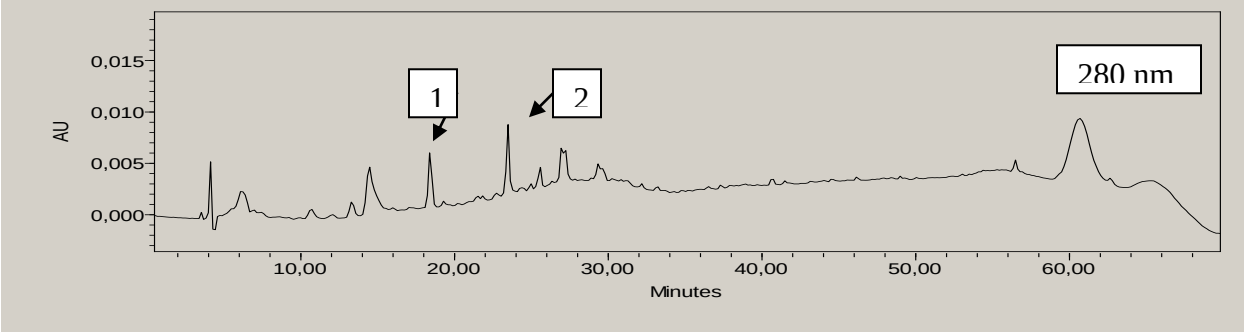


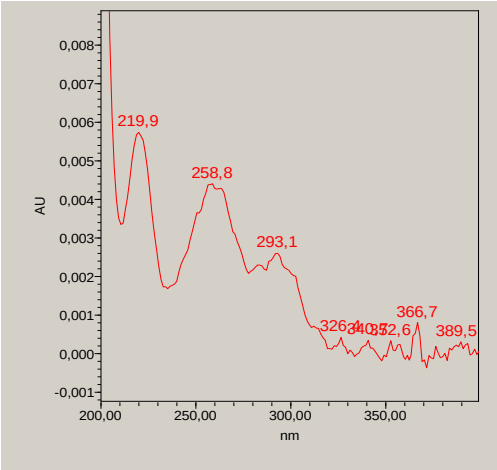
Rt: 24.80, Vitexin-like

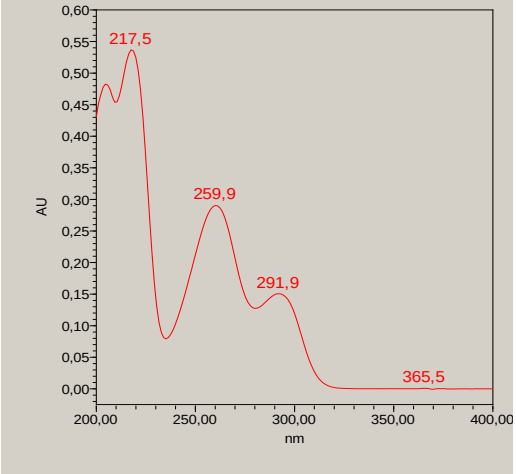
Vitexin, pure substance, Rt: 24.50

1,2,3,4 ……..spectra typical for phenolic acids

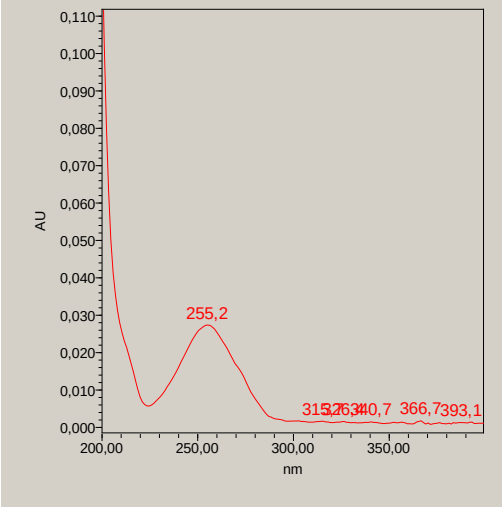
2.1.2. Acid hydrolyzed sample



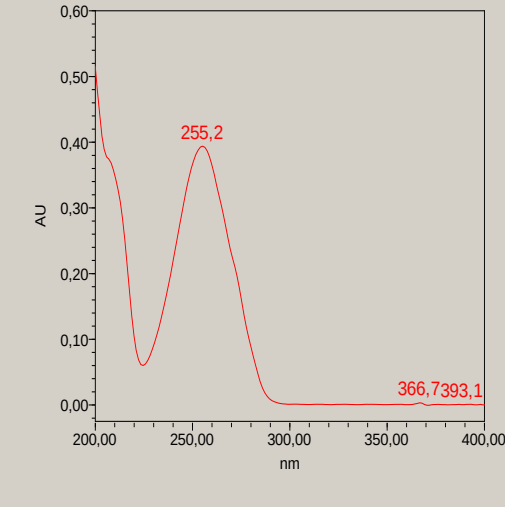




1. Rt: 18.24, Mnium marginatum



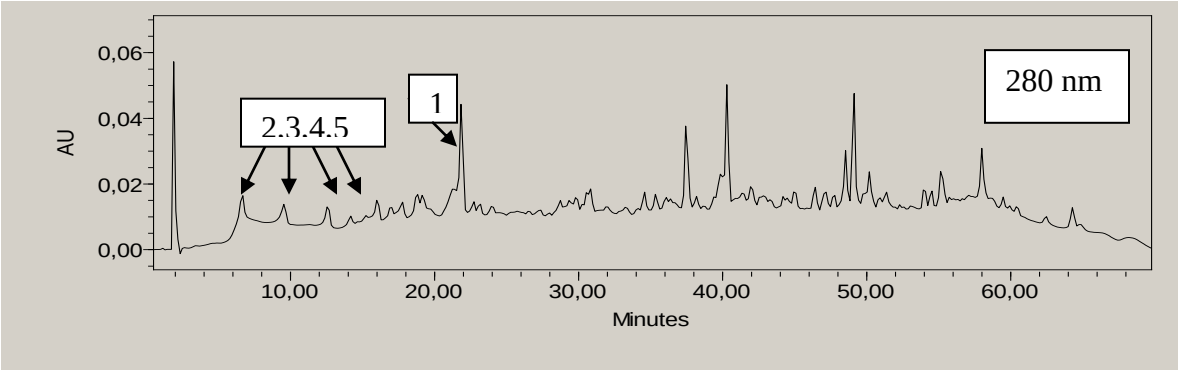
Vanillic ac., pure substance, Rt: 18.3

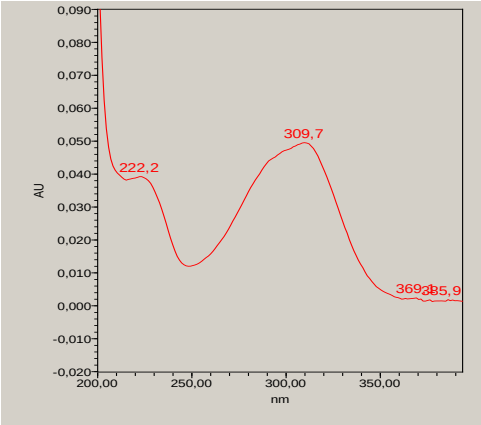


2. Benzoic acid drv., Rt: 23.51

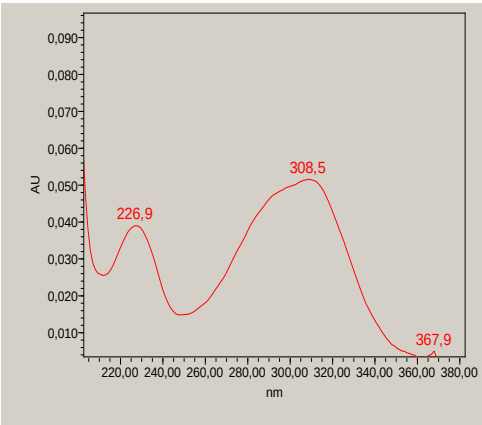
p-Oh benzoic ac., pure substance, Rt: 13.53

2.1.3. Free phenolic acid fraction

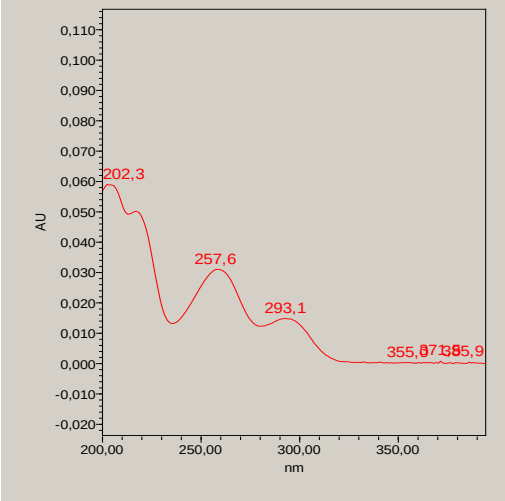




Coumaric acid-like, Rt: 21.69



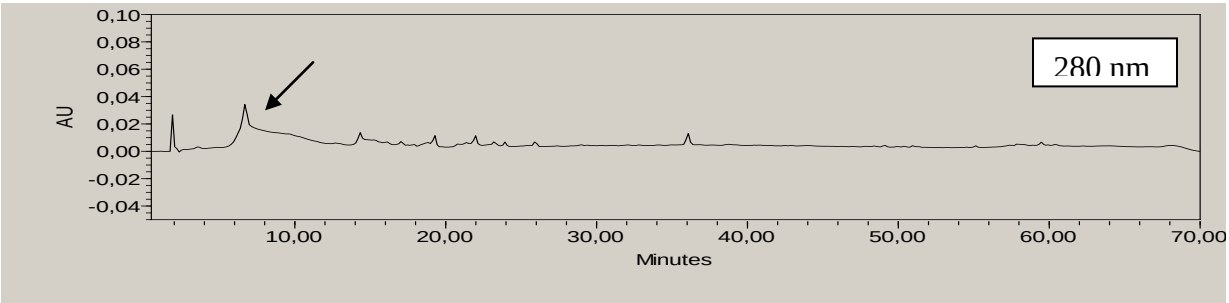
p-coumaric ac., pure substance, Rt: 17.45

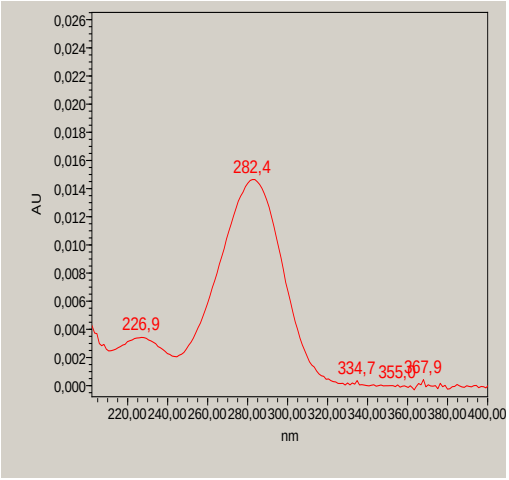


Benzoic acid derivative, Rt: 9.654

2,3,4,5 …….spectra typical for phenolic acid

2.1.4. Acid-hydrolysable phenolic acid fraction



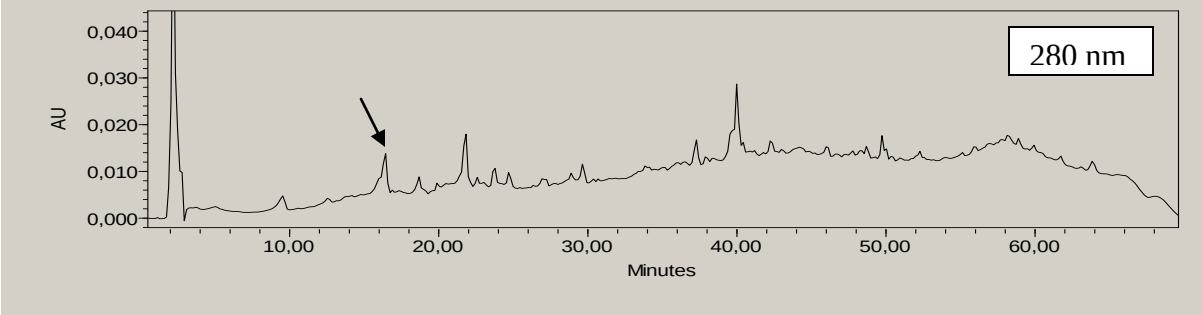


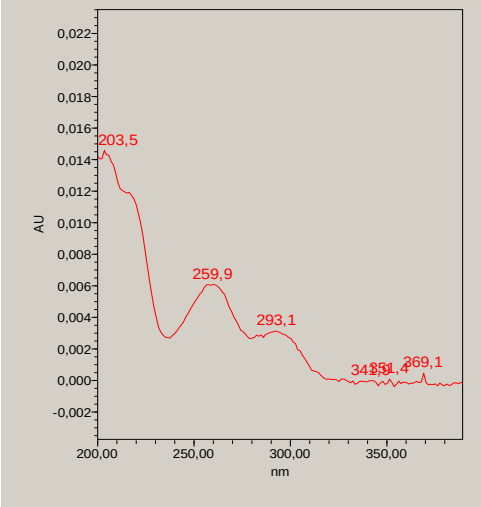
Rt: 6.276 Hesperidin type flavonoid ?

BHPA fraction did not show any results.

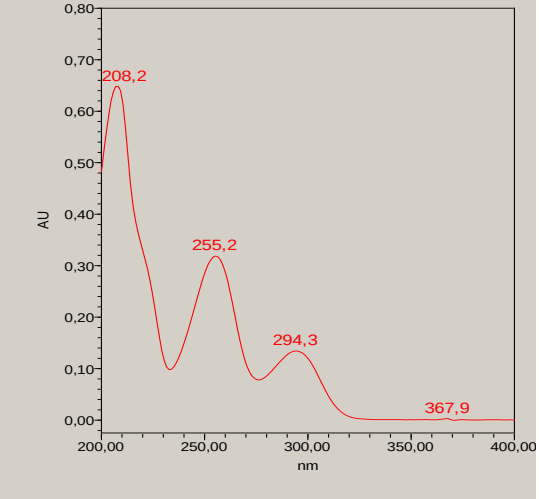
2.2 HPLC analysis of *Leucobryum glaucum*

2.2.1. Nonhydrolyzed sample



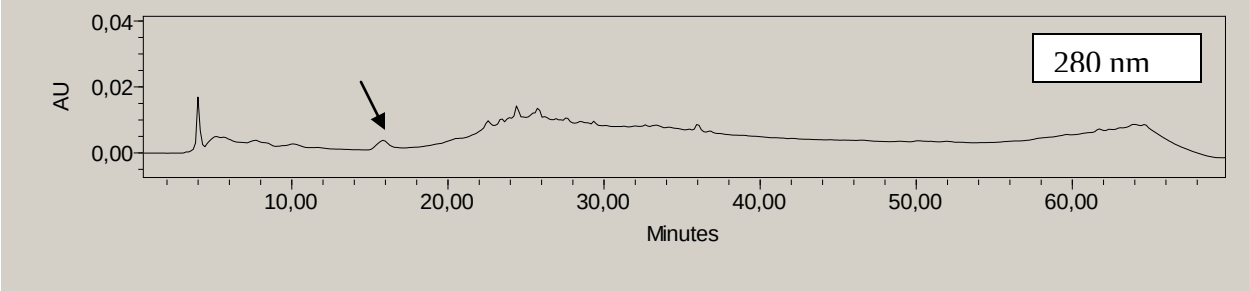


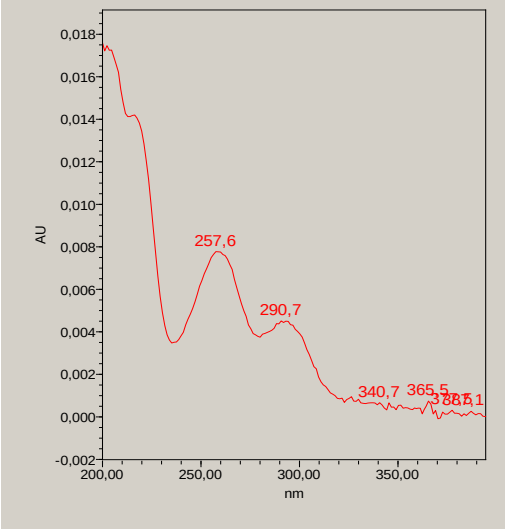
Benzoic acid derivative, Rt: 15.10

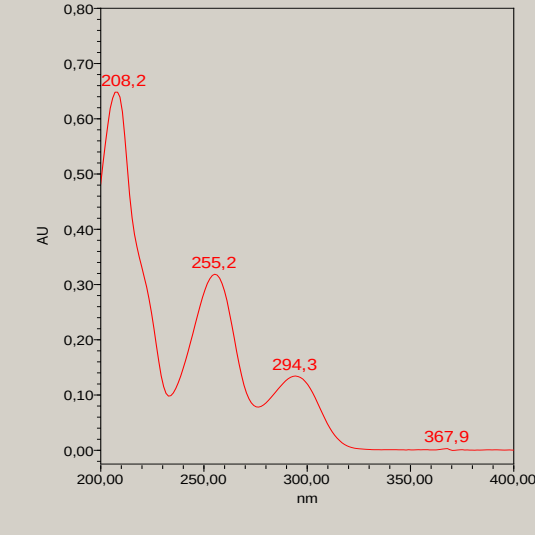


2, 4-di OH benzoic ac, pure substance, Rt: 16.56

2.2.2. Acid hydrolyzed sample



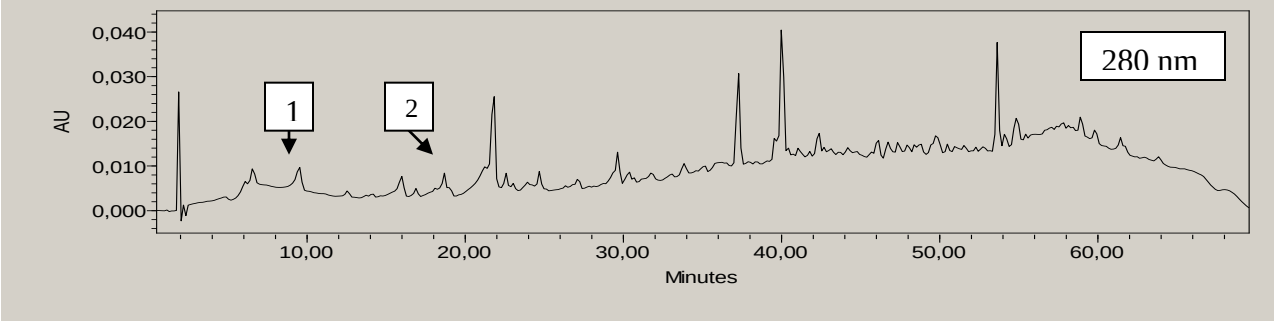


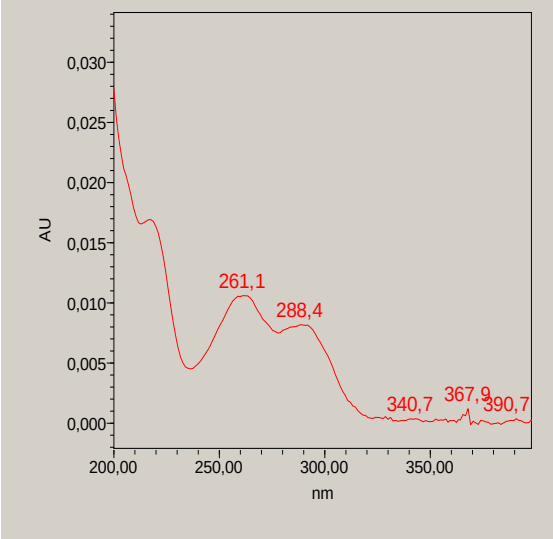


Leucobryum glaucum, Rt: 15.88

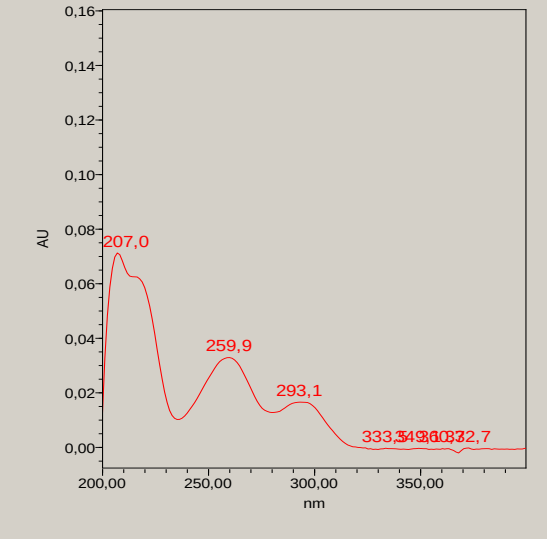
2, 4-di OH benzoic ac, pure substance, Rt: 16.56

2.2.3. Free phenolic acid fraction

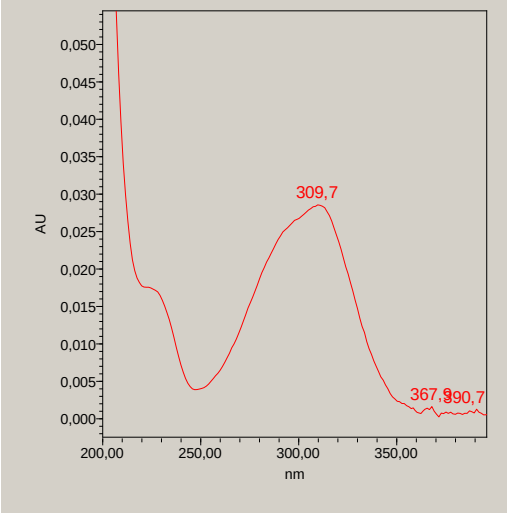




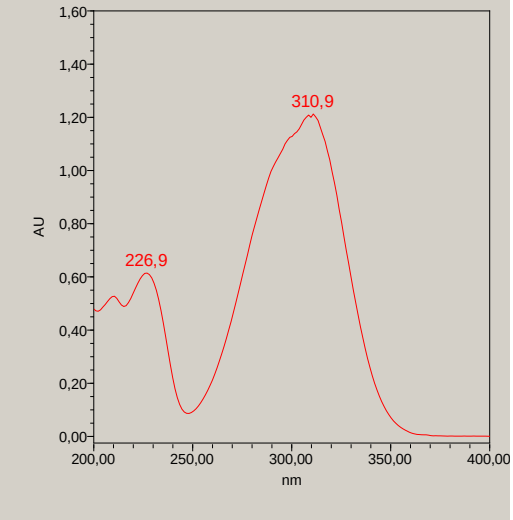
1. Rt: 9.623, *L. glaucum*



3, 4-di OH Benzoic ac., pure substance, Rt: 5.554

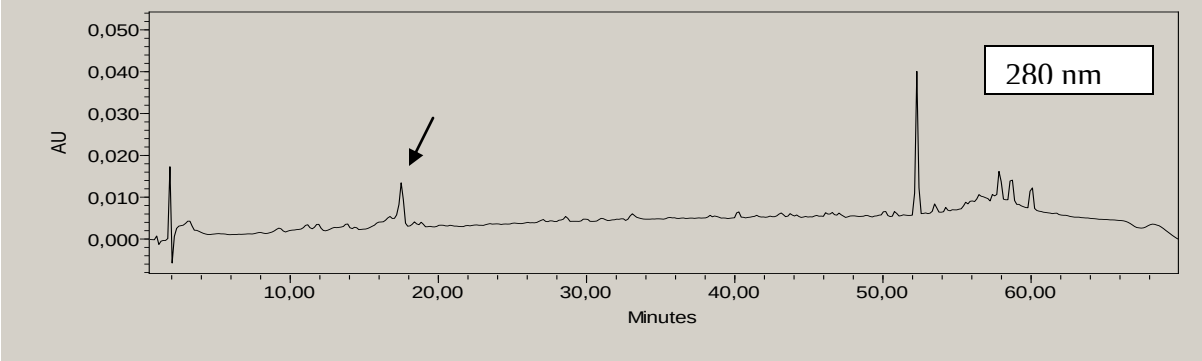


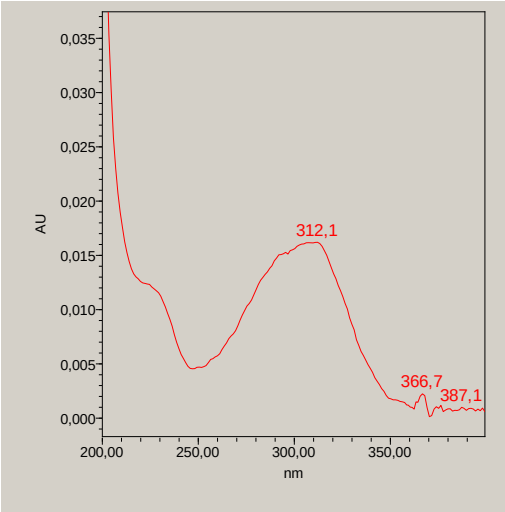
2. *L. glaucum*, Rt: 18.43



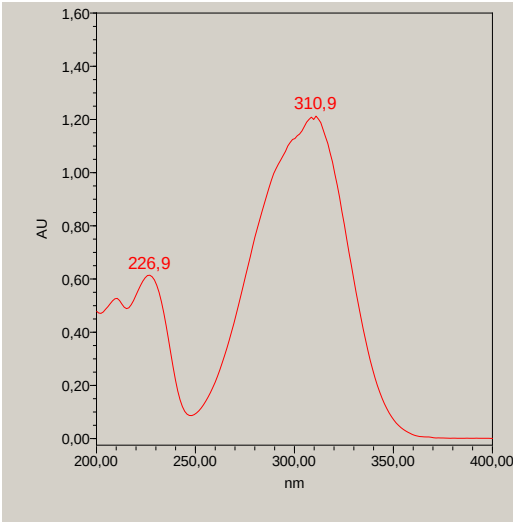
p-coumaric ac., pure substance, Rt: 17.45

2.2.4. Acid-hydrolysable phenolic acids fraction





L. glaucum, Rt: 17.43



p-coumaric ac., pure substance, Rt: 17.45

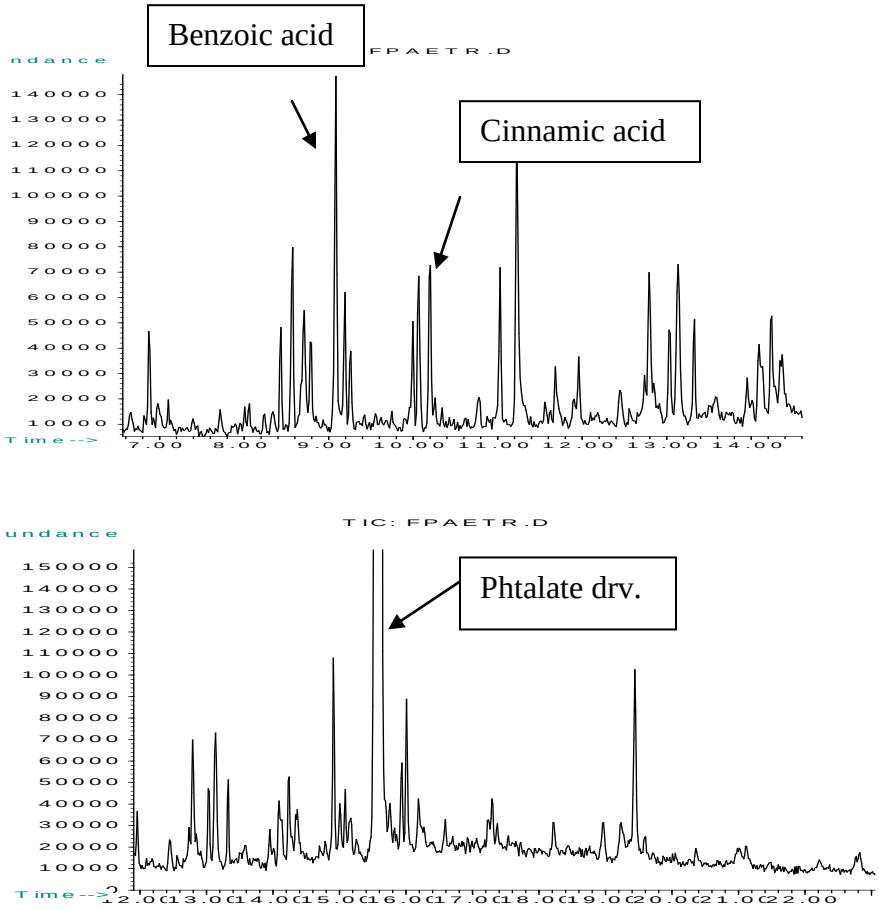
Note: BHPA fraction of both mosses doesn't contain any alkaline-hydrolysable phenolic acids.

3. GC-MS analysis

3.1. GC-MS analysis of *Mnium marginatum*

3.1.1. Free phenolic acids fraction

Rt (min)	Identified compound
6.871	4-OH Benzoic acid – TMS *
8.44	Vanillic acid di – TMS
9.071	Benzoic acid 3,4 bis – TMS
9.997	Hexadecanoic acid, methyl ester
10.20	Cinnamic acid –TMS
11.23	Hexadecanoic acid tri-TMS *
15.43	Phtalate derivative



All GC-MS spectra are in Attachment no. 4.

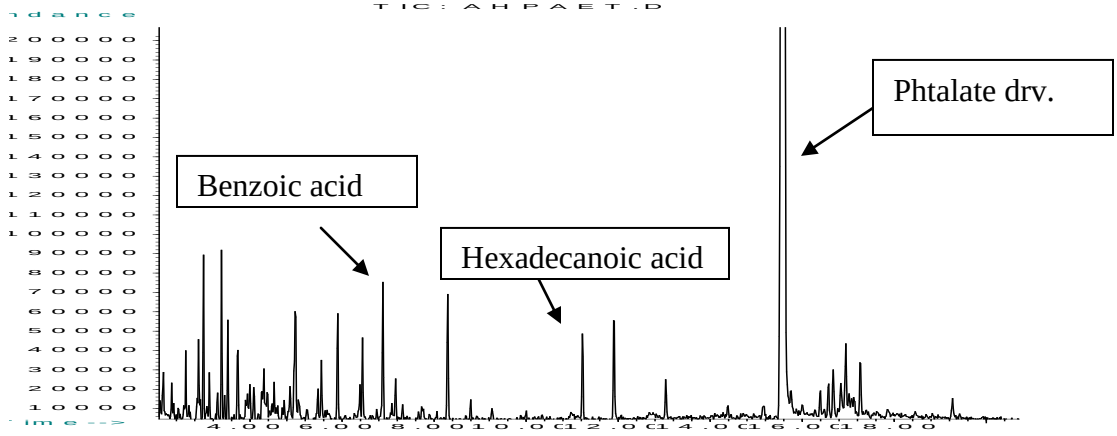
The spectra were compared with those from pure substances and from Wiley library.

3.1.2. Alkaline-hydrolysable phenolic acid fraction

Rt (min)	**Identified compound**
11.067	Methyl-2 ethylhexyl phtalate
11.249	Hexadecanoic acid
15.600	Di-(2-ethylhexyl) phtalate

3.1.3. Acidic-hydrolysable phenolic acid fraction

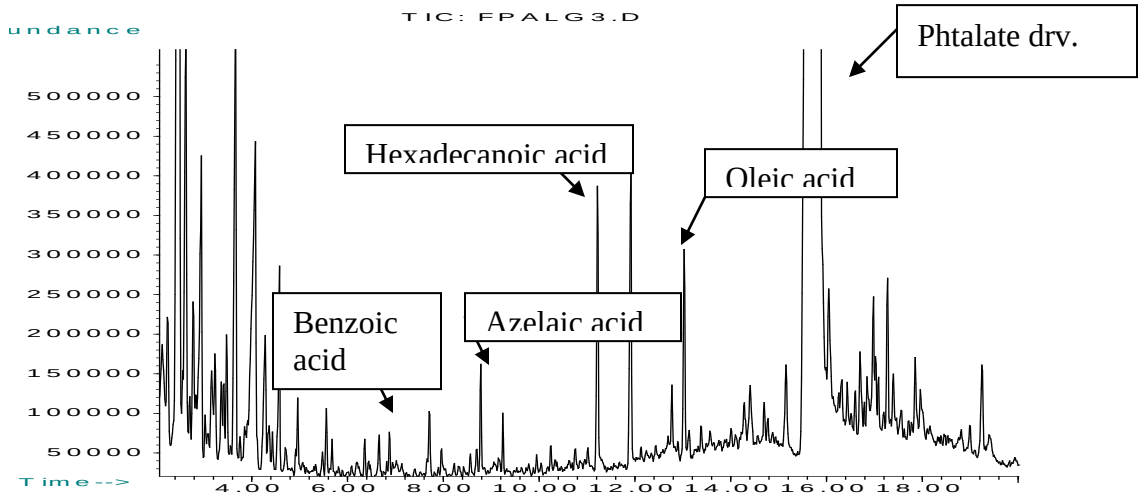
Rt (min)	**Identified compound**
6.889	Benzoic acid – TMS *
11.221	Hexadecanoic acid tri -TMS
15.553	Di (2-ethylhexyl) phtalate



3.2. GC-MS analysis of *Leucobryum glaucum*

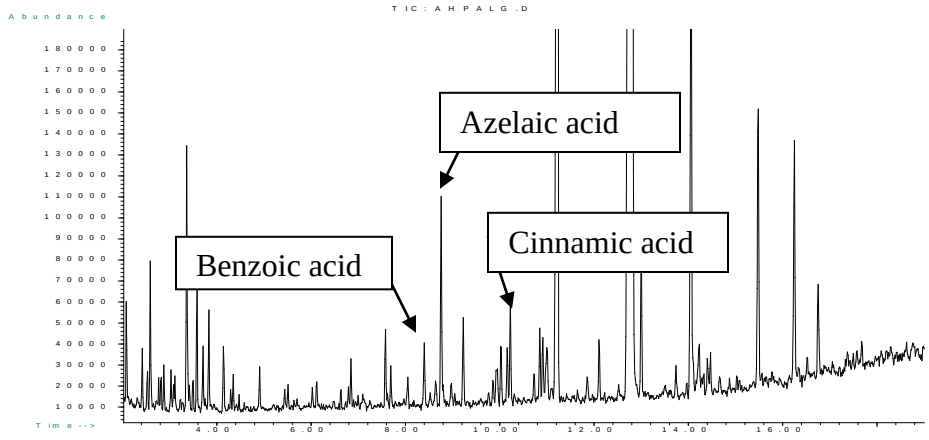
3.2.1. Free phenolic acids fraction

Rt (mins)	Identified compound
6.871	4-OH Benzoic acid -TMS *
8.784	Azelaic acid -TMS
11.217	Hexadecanoic acid -TMS *
12.772	Oleic acid
15.052	Phtalate derivative *



3.2.2. Acidic-hydrolysable phenolic acids fraction

Rt (mins)	Identified compound
8.400	Benzoic acid –TMS*
8.758	Azelaic acid –TMS
10.157	Cinnamic acid -TMS*
11.195	Hexadecanoic acid -TMS*



BHPA fraction didn't contain any alkaline-hydrolysable phenolic acids.

*…..Same compounds in both *M. marginatum* and *L. glaucum*.

3.3. Thiolysis

Mosses didn't showed any presence of procyanidins, but in case of standard – pycnogenol, known for high content of procyanidins we can say, that thiolysis was performed successfully. On both, HPLC and GC-MS spectra were visible thiolysis products – catechins.

3.4. Hydrolyzed and nonhydrolyzed samples

In both cases could be seen some spectra corresponding to flavonoid-like structures or phenolic acids, but the most effective method was procedure according to Kim et al., (Extractable phenolic acids). Most of the phenolic acids were in free form.

V. Discussion

Plants, herbs and some moss species were studied for their antimicrobial activity. The antibiotically active substances of *Atrichum, Dicranum, Mnium, Polytrichum,* and *Sphagnum* spp. are considered to be polyphenolic compounds (McCleary & Walkington, 1966). I have proved that even in species *L. glaucum* and *M. marginatum* were found polyphenolic compounds like phenolic acids and flavonoids responsible for their antioxidant and free radical scavenging activity. Mainly derivatives of benzoic acid and flavonoid vitexin were found.

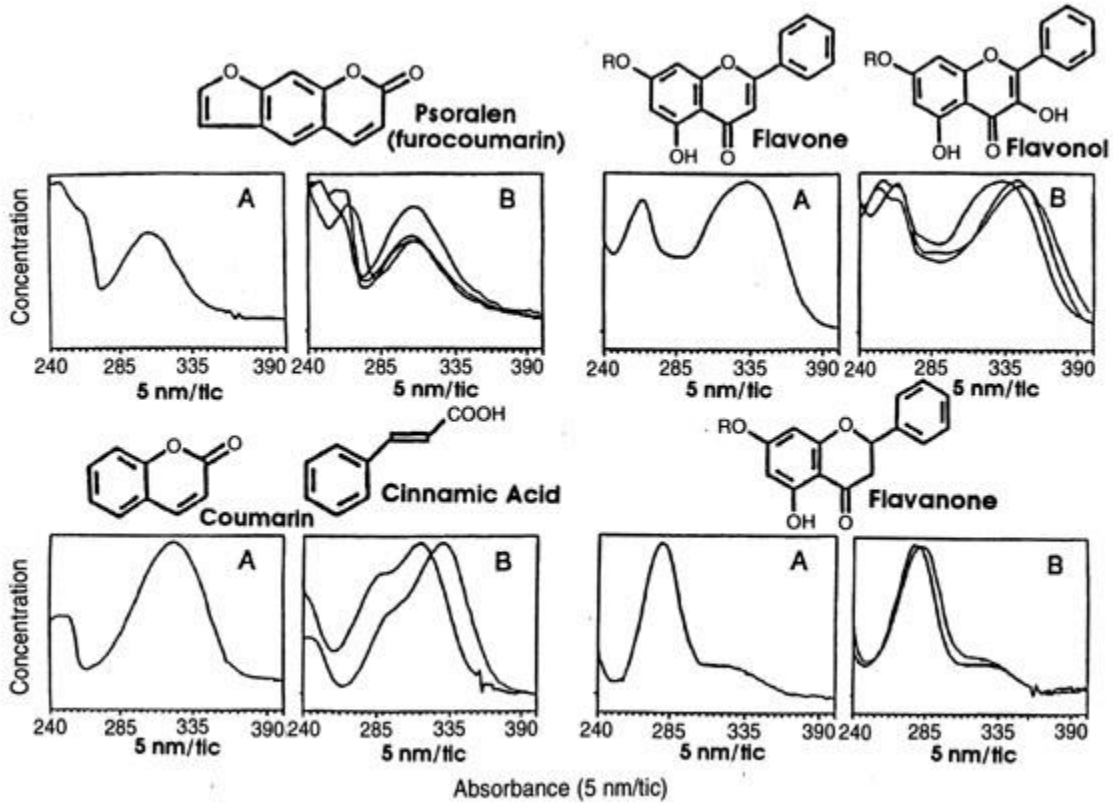


Fig. 1: Phenolic Compounds Produced in Citrus (Berhow et al., 1996)

Flavones from Bryophyta can be subdivided into derivatives of apigenin, luteolin, scutellarein, isoscutellarein, hypolaetin and tricetin (Huneck, 1983). Among the monoflavonoids apigenin, luteolin, kaempferol and orobol derivatives are the usual ones found in mosses (Zinsmeister; Markham). Biflavonoids from apigenin, luteolin and

eryodictiol are also an important source of secondary metabolites from mosses (Geiger; Geiger; Markham; López, 1994).

Total phenolic content was determined using Folin-Ciocalteau reagent. Reducing power, scavenging 1, 1-diphenyl-2-picrylhydrazyl and nitric oxide radicals and inhibition of site-specific and nonsite-specific hydroxyl radical-mediated 2-deody-D-ribose degradation. Caffeic acid was used as positive control for free radical scavenging and antioxidant activity.

HPLC and GC-MS analysis were used to analyze the ethanolic extracts of *L. glaucum* and *M. marginatum* and approved that these extracts contain polyphenolic compounds. The major fragments of spectra and iongrams (TMS-derivatives) of ethanol extracts of mosses were identical with those of standards.

VI. Conclusion

Antioxidant and free radical scavenging activity as well as content determination of five moss species was the aim of this work by using an optimized HPLC and GC-MS method. Total phenolic content, reducing power, antioxidant and free radical scavenging of ethanol extracts of five moss species were determined in vitro. No correlation between the total phenolic content and antioxidant or scavenging activities was found.

1. Antioxidant and free radical scavenging activity of mosses

All tested species of mosses showed scavenging and antioxidant activity, which were lower in comparison with caffeic acid. Extracts od *C. purpureus* and *D. polysetum* showed no effects on nonsite-specific hydroxyl radical-mediated 2-deoxy-D-ribose degradation in the range of the studies concentrations. Since these extracts inhibited 2-deoxy-D-ribose degradation in the site-specific variant of the assay, we assume that they should inhibit hydroxyl radical formulation by chelating and deactivating iron ions. However, the effects of the tested moss extracts were not significantly correlated with the total phenolic content (α=0, 05). Nevertheless, it has to be noted that various constituents with synergistic or antagonistic effects could modulate the final activity.

Table 1

Total phenolic content, reducing power and 50 % effective concentrations (EC_{50}) of antioxidant activities of ethanol extract of the mosses in comparison with caffeic acid.

Moss	TPC (%)	RP (mg/ml)	DPPH (mg/ml)	NO (mg/ml)	SSOH (mg/ml)	NSSOH (mg/ml)
C. purpureus	3.886±0.011	5.661±0.968	0.487±0.001	0.310±0.016	1.974±0.553	> 4.000
D. polysetum	2.553±0.006	4.891±0.836	0.299±0.016	0.279±0.020	2.359±0.122	> 4.000
D. scoparium	3.432±0.006	4.492±0.768	0.170±0.001	0.206±0.012	0.746±0.368	3.197±2.131
L. glaucum	3.781±0.007	21.849±5.521	0.299±0.020	0.363±0.002	2.392±0.001	1.254±1.656
M.marginatum	2.581±0.003	10.528±1.841	0.208±0.016	0.226±0.012	0.637±0.580	1.080±0.907
Caffeic acid	-	-	0.002±0.001	0.008±0.001	0.008±0.001	0.003±0.001

All presented values were calculated from the experiment repetitions at least reported with ± 95% confidence limits.

Chobot et al, 2006. Article was published in Fitoterapia, 2006.

2. HPLC and GC-MS analysis

Ethanolic extracts of *L.glaucum* and *M.marginatum* were analyzed by HPLC and GC-MS analysis. These analyses showed that both mosses contained phenolic acids as well as flavonoid- like structures. HPLC analysis of extracts showed some spectra typical for flavonoid vitexin (*Mnium marginatum)* and phenolic acids (both mosses), most probably benzoic acid derivatives. Their retention times slightly differ from those of pure substances, but it might be due to low concentration of these compounds. We confirmed HPLC results with GC-MS spectra, where the results were clearer and proved that mosses really do contain various types of phenolic acids. I supposed that these members of polyphenolic family are responsible for antioxidant and free radical scavenging activity of mosses.

VII. Abstract

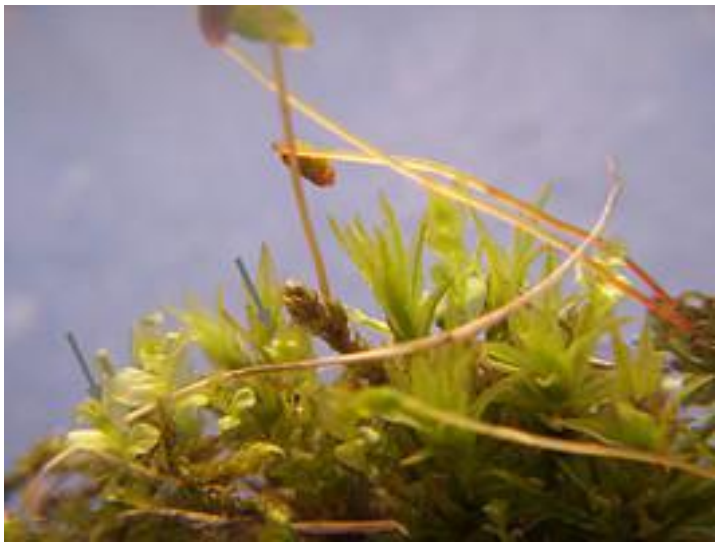
The chemical composition and antioxidant activity of ethanolic extracts of five moss species are presented in this diploma thesis. The total phenol content was estimated as gallic acid equivalents by the Folin-Ciocalteu reagent method, while the qualitative composition of the extracts were determined by high performance liquid chromatography coupled with photodiode array detection and by gas chromatography – mass spectroscopy detection. The antioxidant properties assessed included iron(III) reduction, 1, 1-diphenyl-2-picrylhydrazyl anion free radical scavenging and the ability of extracts to protect 2-deoxy-D-ribose against hydroxyl radical-mediated degradation was assessed. The extracts contained phenolic compound. Free phenolic acids as benzoic acid derivatives and glycosides as vitexin-like structures.The ethanolic extracts of five moss species demonstrated antioxidant and free radical scavenging activity; however, they were not as potent as the positive control.

VIII. Abstract in Czech

V této práci jsem se zaměřila na pět druhů mechů- *C. purpureus*, *D. scoparium*, *D. polysetum*, *L. glaucum*, *M. marginatum*, u kterých jsem zkouala jejich antioxidační aktivitu a u dvou vybraných meších- *L. glaucum* a *M. marginatum* i jejich složení. Celkový obsah fenolických látek byl stanoven pomocí Folin- Ciocalteova reagentu, zatímo kvalitativní kompozice etanolických extraktů byla determinována HPLC a GC-MS analýzou. Zjišťovala jsem antioxidační vlastnostnosti, které zahrnovaly redukci železa(III), vychytávání volného radikálového anionu 1,1-difenyl-2-pikrylhydrazylu a schopnost extraktu zamezit degradaci 2-deoxy-D-ribosy. Extrakty obsahují polyfenolické látky, zejména pak deriváty kyseliny benzoové a dale pak flavonoidy vitexinového typu. Extrakty všech pěti mechů vykazují antioxidační vlastnosti a schopnost vychytávat volné radikály, i když v porovnání se standardem- kyselinou kávovou, byly výsledky nižší.

IX. Attachments

Attachment no. 1

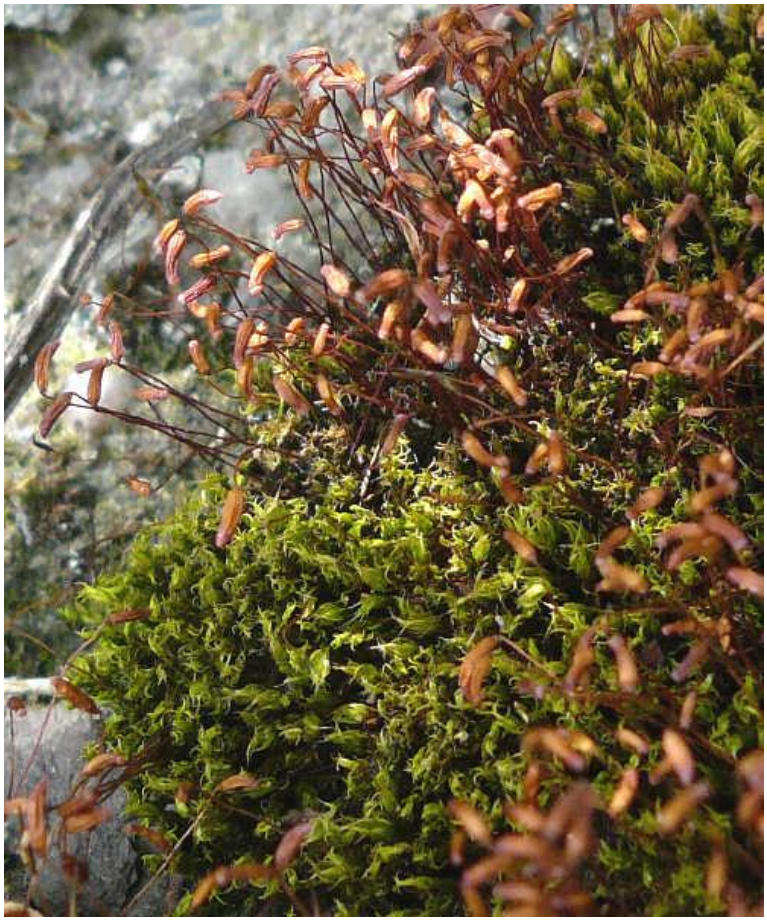


Mnium marginatum

Leucobryum glaucum

Dicranum scoparium

Dicranum polysetum



Ceratodon purpureus

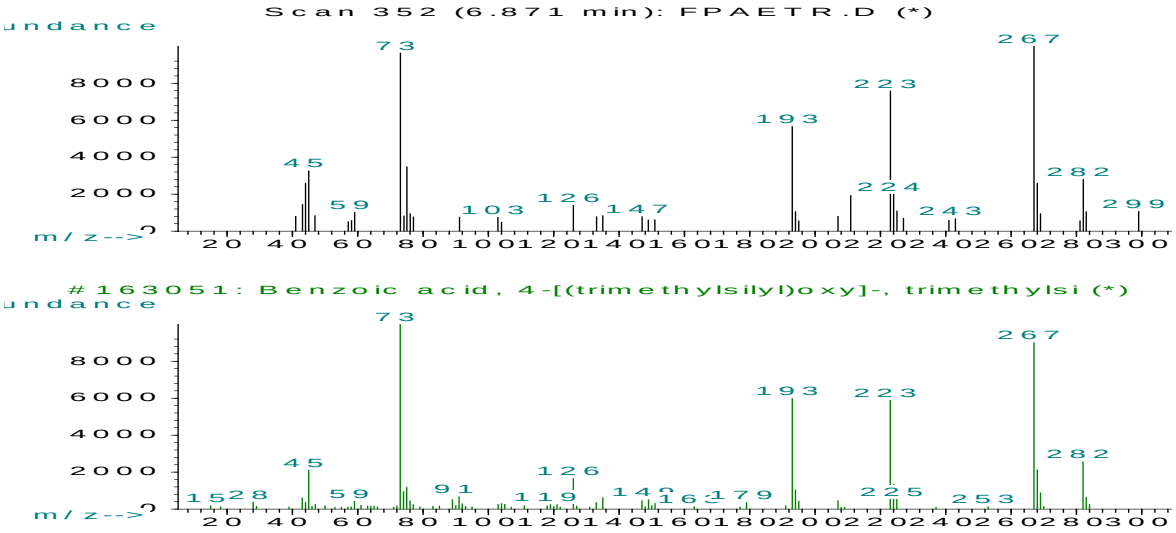
Pictures were taken from www.biolib.cz web sites.

Attachment no. 2.

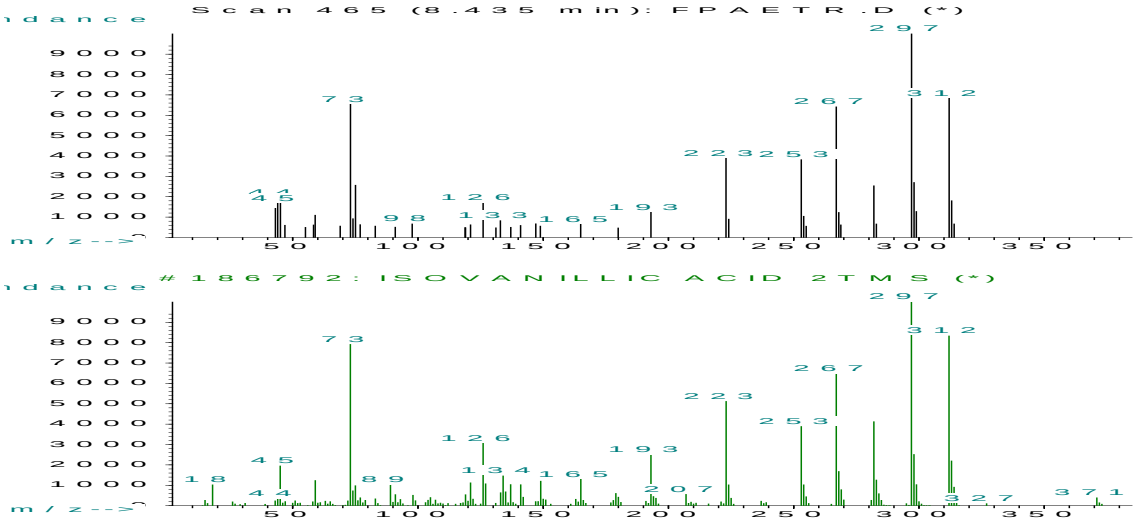
GC-MS spectra were compared with those obtained from analyses of pure substances during the study, and with those from Division's own library compounds, their methyl esters or trimethylsilyl (TMS) derivatives, and from Wiley 275 L library. The matching with the library spectra, if expressing in percentages, has been between 97-99%.

GC-MS analysis

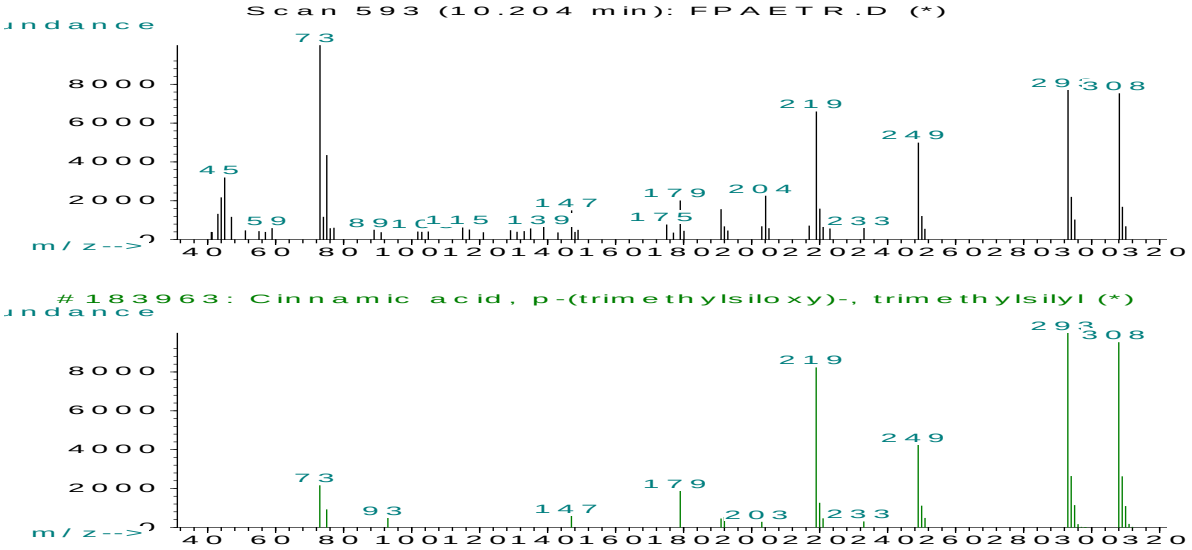
*Mnium marginatum***,** Free phenolic acids



4-OHBenzoic acid-TSM

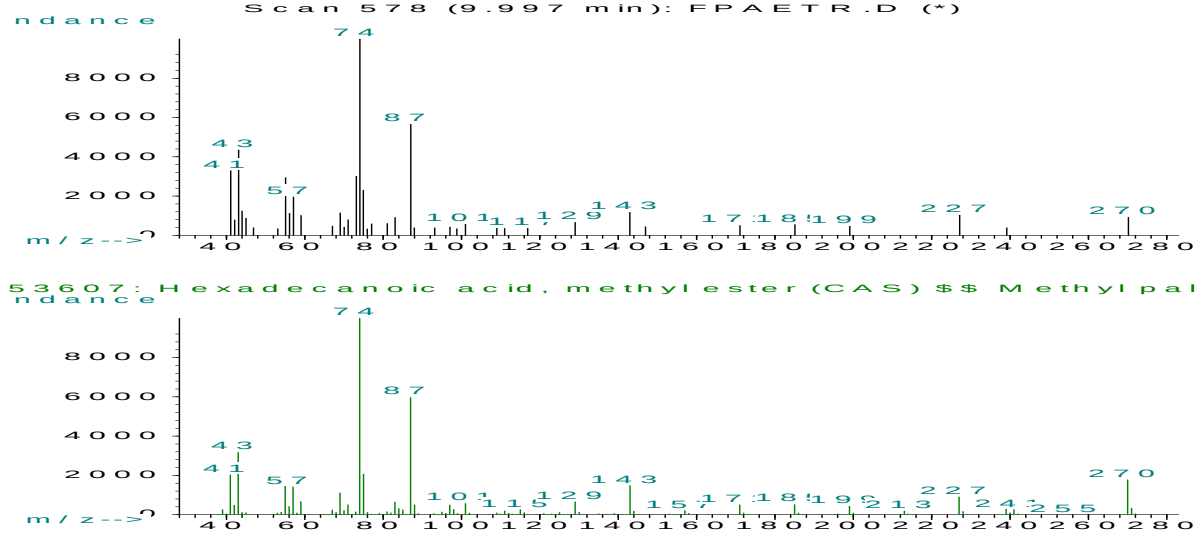


Vanillic acid derivative-TSM



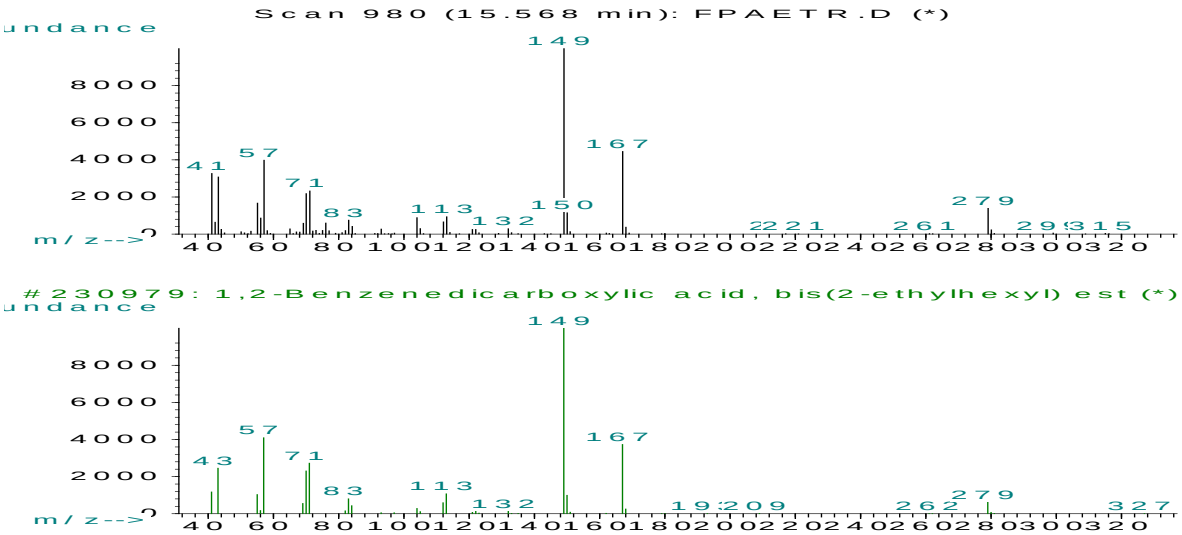
Cinnamic acid-TSM

Fatty acids



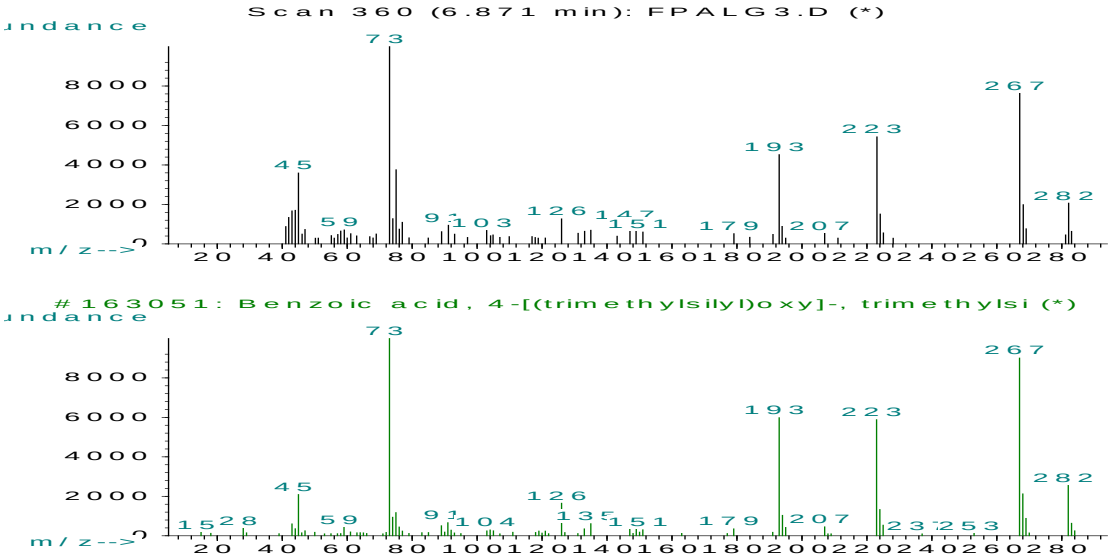
Hexadecanoic acid

Dicarboxylic acids



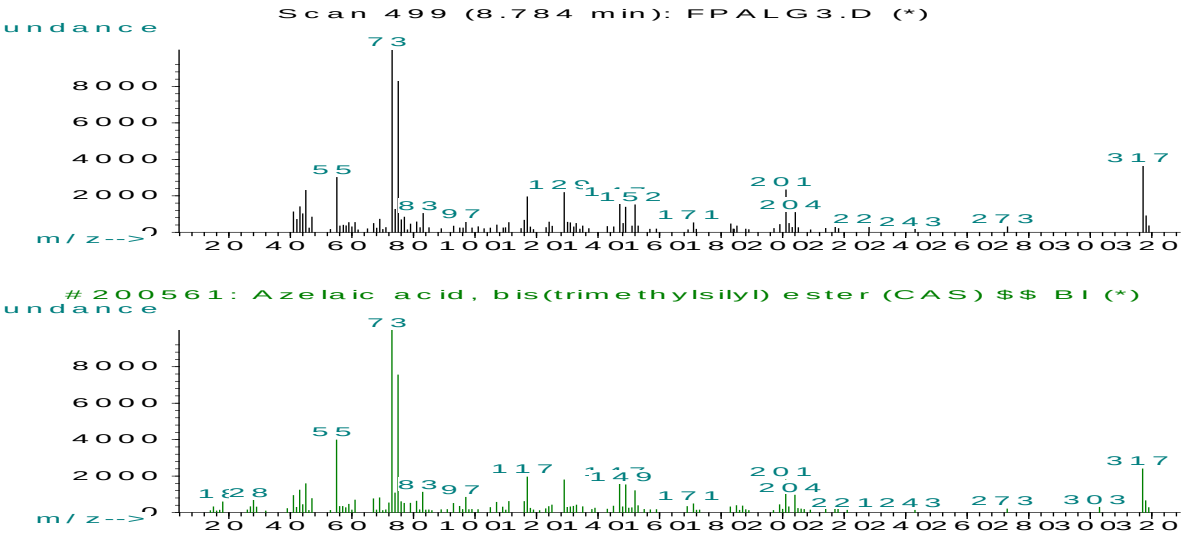
Phtalate drv.

Leucobryum glaucum, Free phenolic acid fraction (FPA)



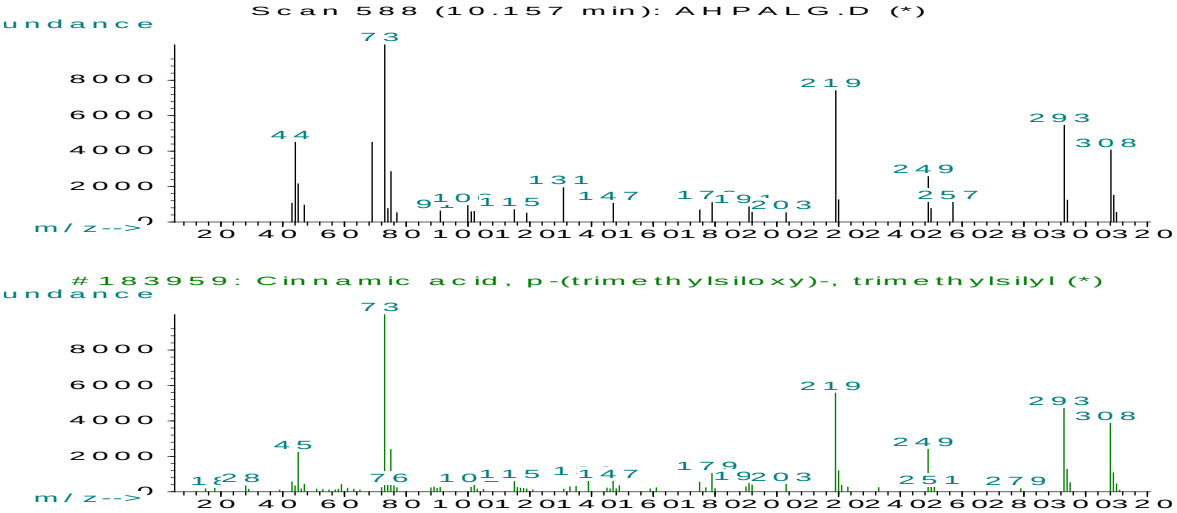
Benzoic acid-TSM

Dicarboxylic acids



Azelaic acid-TSM (Nonanedioic acid-TSM)

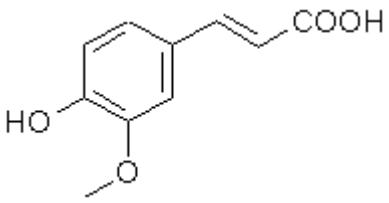
Leucobryum glaucum, Acid-hydrolysable phenolic acids



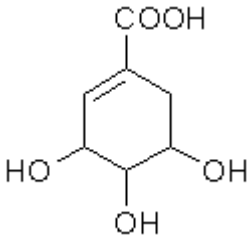
Cinnamic acid-TSM

Attachment no. 3.

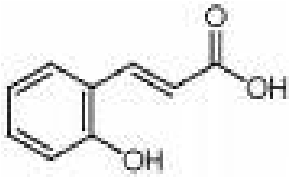
Phenolic acids

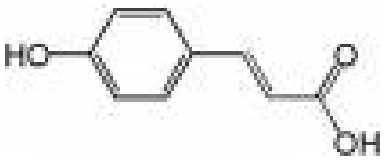


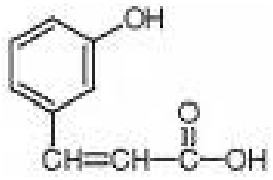
Ferulic acid



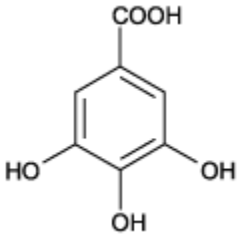
Shikimic acid





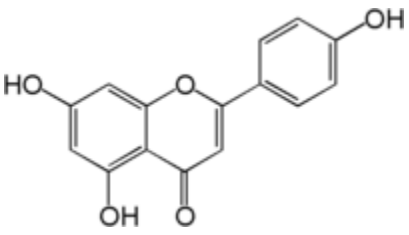


***trans o*-Coumaric acid** ***p*-Coumaric acid** ***trans m*-Coumaric acid**

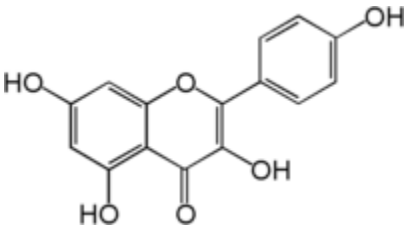


Gallic acid

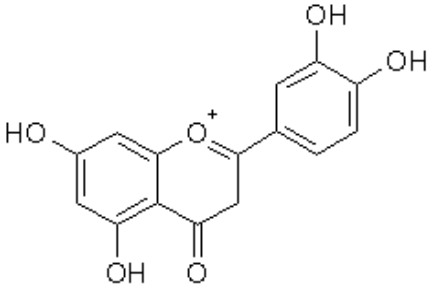
Flavonoid aglycones



Apigenin

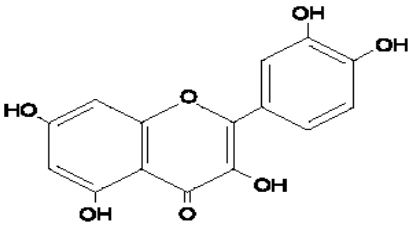


Kaempferol

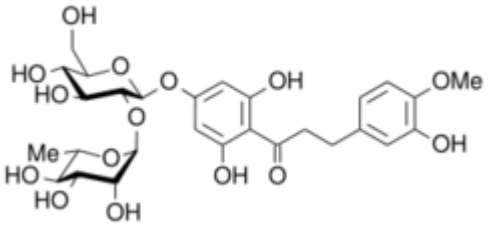


Luteolin

Quercetin

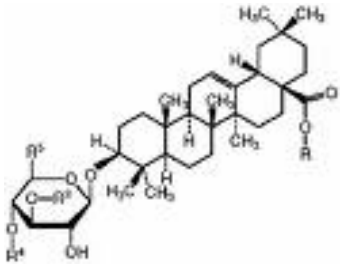


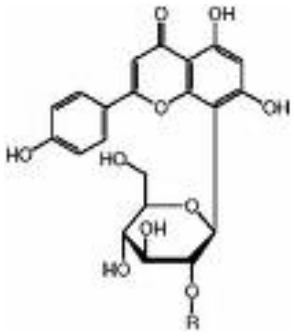
Flavonoid glycosides



Hesperidin(Hesperitin-7-O-rhamnoside(1-6)-glucoside)

Hyperoside(Quercetin-3-O-galactoside)





Vitexin (Apigenin-8-C-glucoside)

Attachment no. 4

Griess method

Scavenging of nitric oxide radicals

Caffeic acid

mg/ml	sample	blind sample	conc - log	difference
0.36	0.109	0.009	0.443697	0.1
0.2	0.104	0.006	0.69897	0.098
0.08	0.135	0.005	1.09691	0.13
0.036	0.137	0.011	1.443697	0.126
0.032	0.136	0.008	1.49485	0.128
0.028	0.15	0.011	1.552842	0.139
0.024	0.149	0.008	1.619789	0.141
0.016	0.159	0.008	1.79588	0.151
0.012	0.17	0.01	1.920819	0.16
0.008	0.215	0.01	2.09691	0.205
0.004	0.29	0.01	2.39794	0.28
0.0036	0.309	0.014	2.443697	0.295
0.0032	0.321	0.014	2.49485	0.307
0.0028	0.336	0.013	2.552842	0.323
0.0024	0.351	0.005	2.619789	0.346
0.0016	0.413	0.009	2.79588	0.404
0.0012	0.45	0.008	2.920819	0.442
0.0008	0.492	0.006	3.09691	0.486
0.0004	0.509	0.009	3.39794	0.5
0.00036	0.517	0.005	3.443697	0.512
0.00032	0.511	0.004	3.49485	0.507
0.00028	0.516	0.009	3.552842	0.507
0.00024	0.527	0.008	3.619789	0.519
0.0002	0.522	0.005	3.69897	0.517
0	0.51			0.5125
0	0.515			

1.2.1. Mosses

Dicranum polysetum

mg/ml	sample	Blind sample	Conc-log	difference
1	0.465	0.435	0	0.03
0.889	0.381	0.355	0.051098	0.026
0.778	0.301	0.246	0.10902	0.055
0.667	0.287	0.195	0.175874	0.092
0.444	0.23	0.058	0.352617	0.172
0.333	0.263	0.035	0.477556	0.228
0.222	0.33	0.039	0.653647	0.291
0.111	0.409	0.013	0.954677	0.396
0.1	0.405	0.01	1	0.395
0.0889	0.409	0.007	1.051098	0.402
0.0778	0.417	0.009	1.10902	0.408
0.0667	0.408	0.002	1.175874	0.406
0.0444	0.406	0	1.352617	0.406
0.0333	0.434	0.003	1.477556	0.431
0.0222	0.429	0.002	1.653647	0.427
0.0111	0.46	0.005	1.95677	0.455
0.01	0.497	0.003	2	0.494
0.00889	0.5	0.002	2.051098	0.498
0.00778	0.528	0.001	2.10902	0.527
0.00667	0.513	0.002	2.175874	0.511
0.00556	0.502	-0.003	2.254925	0.505
0.00444	0.5	0	2.352617	0.5
0	0.525			0.52
0	0.515			

Ceratodon purpureus:

mg/ml	sample	blind sample	conc- log	difference
1	0.84	0.703	0	0.137
0.889	0.73	0.573	0.051098	0.157
0.778	0.623	0.517	0.10902	0.106
0.667	0.641	0.513	0.175874	0.128
0.444	0.411	0.194	0.352617	0.217
0.333	0.332	0.064	0.477556	0.268
0.222	0.369	0.028	0.653647	0.341
0.111	0.452	0.006	0.954677	0.446
0.1	0.471	0.006	1	0.465
0.0889	0.474	0	1.051098	0.474

0.778	0.477	0.002	1.151098	0.475
0.0667	0.504	0.015	1.175874	0.489
0.0444	0.524	0.005	1.352617	0.519
0.0333	0.544	0.005	1.477556	0.539
0.0222	0.552	0.001	1.653647	0.551
0.0111	0.587	0	1.954677	0.587
0.01	0.6	0.002	2	0.598
0.00889	0.611	0.004	2.051098	0.607
0.00778	0.621	0.002	2.10902	0.619
0.00667	0.635	0.005	2.175874	0.63
0.00556	0.648	0.001	2.254925	0.647
0.00444	0.647	0	2.352617	0.647
0	0.637			0.647
0	0.657			

Dicranum scoparium:

mg/ml	sample	blind sample	conc-log	difference
1	0.466	0.399	0	0.067
0.889	0.364	0.218	0.051098	0.146
0.778	0.255	0.105	0.10902	0.15
0.667	0.295	0.061	0.175874	0.234
0.444	0.283	0.037	0.352617	0.246
0.333	0.301	0.035	0.477556	0.266
0.222	0.35	0.02	0.653647	0.33
0.111	0.43	0.003	0.954677	0.427
0.1	0.476	0.029	1	0.447
0.0889	0.481	0.014	1.051098	0.467
0.0778	0.504	0.013	1.10902	0.491
0.0667	0.505	0.001	1.175874	0.504
0.0444	0.559	0.006	1.352617	0.553
0.0333	0.585	0.002	1.477556	0.583
0.0222	0.633	0.005	1.653647	0.628
0.0111	0.64	0.01	1.954677	0.63
0.01	0.634	0.009	2	0.625
0.00889	0.638	0.01	2.051098	0.628
0.00778	0.664	0.002	2.10902	0.662
0.00667	0.707	0.001	2.175874	0.706
0.00556	0.738	0.005	2.254925	0.733
0	0.739			0.747
0	0.755			

Leucobryum glaucum:

mg/ml	sample	blind sample	conc-log	different
1	0.423	0.276	0	0.147
0.889	0.39	0.258	0.051098	0.132
0.778	0.359	0.219	0.10902	0.14
0.667	0.282	0.15	0.175874	0.132
0.444	0.25	0.108	0.352617	0.142
0.333	0.215	0.05	0.477556	0.165
0.222	0.25	0.04	0.653647	0.21
0.111	0.303	0.022	0.954677	0.281
0.1	0.316	0.02	1	0.296
0.0889	0.324	0.02	1.051098	0.304
0.0778	0.325	0.011	1.10902	0.314
0.0667	0.338	0.008	1.175874	0.33
0.0444	0.374	0.019	1.352617	0.355
0.0333	0.396	0.015	1.477556	0.381
0.0222	0.416	0.014	1.653647	0.402
0.0111	0.418	0.01	1.954677	0.408
0.01	0.4407	0.005	2	0.402
0.00889	0.411	0.001	2.051098	0.41
0.00778	0.428	0.002	2.10902	0.426
0.00667	0.457	0.005	2.175874	0.452
0.00556	0.445	0.001	2.254925	0.444
0	0.441			0.4485
0	0.456			

Mnium marginatum:

mg/ml	sample	blind sample	conc-log	Difference
1	0.441	0.222	0	0.219
0.889	0.354	0.142	0.051098	0.212
0.778	0.284	0.123	0.10902	0.161
0.667	0.193	0.085	0.175874	0.108
0.444	0.202	0.054	0.352617	0.148
0.333	0.215	0.033	0.477556	0.182
0.222	0.238	0.015	0.653647	0.223
0.111	0.299	0.01	0.9546677	0.289
0.1	0.304	0.007	1	0.297
0.0889	0.31	0.01	1.051098	0.3
0.0778	0.32	0.005	1.10902	0.315
0.0667	0.346	0.007	1.175874	0.339
0.0444	0.378	0.002	1.352617	0.376

0.0333	0.408	0.008	1.477556	0.4
0.0222	0.43	0.006	1.653647	0.424
0.0111	0.487	0.001	1.954677	0.486
0.01	0.501	0.004	2	0.497
0.00889	0.515	0.003	2.051098	0.512
0.00778	0.524	0.001	2.10902	0.523
0.00667	0.32	0.005	2.175874	0.527
0.00556	0.543	0.005	2.254925	0.538
0.00333	0.544	0.001	2.477556	0.543
0	0.54			0.551667
0	0.55			
0	0.565			

Reducing power

Quercetin:

mg/ml	sample1 A	sample2 A	blind s	average	conc-log	difference A
1	2.28	2.301	0.064	2.2905	0	2.2265
0.8	2.29	2.223	0.028	2.2565	0.09691	2.2285
0.4	2.101	2.18	0.013	2.1405	0.39794	2.1275
0.2	2.039	2.063	0.006	2.051	0.69897	2.045
0.1	1.447	1.567	0.008	1.507	1	1.499
0.08	1.32	1.321	0.003	1.3205	1.09691	1.3175
0.06	1.056	1.066	0.004	1.061	1.221849	1.057
0.04	0.756	0.757	0.001	0.7565	1.39794	0.7555
0.02	0.398	0.402	0.001	0.4	1.69897	0.399
0.01	0.313	0.314	0.005	0.3135	2	0.3085
0.008	0.297	0.296	0.007	0.2965	2.0961	0.2895
0.006	0.267	0.264	0	0.655	2.221849	0.2655
0.004	0.247	0.243	0.003	0.245	2.39794	0.242
0.002	0.233	0.2	0	0.2165	2.69897	0.2165
0	0.163	0.165	0.003	0.164		0.161

Caffeic acid:

mg/ml	sample 1	sample 2	blind s	average	conc-log	difference
1	2.306	2.301	0.026	2.3035	0	2.2775
0.8	2.301	2.306	0.024	2.3035	0.09691	2.2795
0.4	2.293	2.29	0.02	2.2915	0.39794	2.2715
0.2	2.205	2.207	0.02	2.206	0.69897	2.186
0.1	1.599	1.603	0.01	1.601	1	1.591
0.08	1.35	1.343	0.004	1.3465	1.09691	1.3425
0.06	1.074	1.072	0.003	1.073	1.221849	1.07

0.04	0.765	0.768	0.003	0.7665	1.39794	0.7635
0.02	0.404	0.407	0.003	0.4055	1.69897	0.4025
0.01	0.301	0.303	0	0.302	2	0.302
0.008	0.272	0.303	0.001	0.2875	2.09691	0.2865
0.006	0.249	.246	0.004	0.2475	2.221849	0.2435
0.004	0.231	0.224	0.003	0.2275	2.39794	0.2245
0.002	0.191	0.197	0.002	0.194	2.69897	0.192
0.001	0.188	0.178	0.008	0.183	3	0.175
0.0008	0.172	0.18	0.008	0.176	3.09691	0.168
0.0006	0.175	0.172	0.002	0.1735	3.221849	0.1715
0.0004	0.162	0.17	0.004	0.166	3.39794	0.162
0.0002	0.167	0.171	0.001	0.169	3.69897	0.168
0	0.165	0.167	0.001	0.166	……..	0.165

Dicranum polysetum:

mg/ml	sample1	sample2	blind s	average	conc-log	difference
5	0.731	0.741	0.444	0.736	-0.69897	0.292
2	0.474	0.491	0.205	0.4825	-0.30103	0.2775
1	0.333	0.335	0.077	0.334	-0	0.257
0.5	0.228	0.231	0.049	0.2295	0.30103	0.1805
0	0.151	0.158	0.002	0.1545		0.1525

Ceratodon purpureus:

mg/ml	sample1	sample2	blind s	average	conc-log	difference
5	0.802	0.839	0.434	0.8205	-0.69897	0.3865
2	0.673	0.67	0.355	0.6715	-0.30103	0.3165
1	0.47	0.456	0.2	0.463	0	0.263
0.5	0.33	0.355	0.109	0.3425	0.30103	0.2335
0	0.159	0.165	0.003	0.162		0.159

Dicranum scoparium:

mg/ml	sample1	sample2	blind s	average	conc-log	difference
5	1.464	1.476	1.022	1.47	-0.69897	0.448
2	0.885	0.876	0.545	0.8805	-0.30103	0.3355
1	0.531	0.534	0.279	0.5325	0	0.2535
0.5	0.334	0.348	0.129	0.341	0.30103	0.212
0	0.159	0.165	0.003	0.162		0.159

Leucobryum glaucum:

mg/ml	sample1	sample2	blind s	average	conc-log	difference
5	1.465	1.441	0.773	1.453	-0.69897	0.69
2	0.901	0.907	0.453	0.904	-0.30103	0.451
1	0.568	0.559	0.245	0.5635	0	0.3185
0.5	0.34	0.334	0.107	0.337	0.30103	0.23
0	0.17	0.17	0.005			

Mnium marginatum:

mg/ml	sample1	sample2	blind s	average	conc-log	difference
5	0.913	0.898	0.325	0.9055	-0.69897	0.5805
2	0.565	0.567	0.173	0.566	-0.30103	0.393
1	0.372	0.375	0.085	0.3735	0	0.2885
0.5	0.275	0.267	0.04	0.271	0.30103	0.231
0	0.17	0.17	0.005	0.17		0.165

DPPH

Caffeic acid

dilution	Mg/ml	sample	Blind s	Conc-log	difference
1.4 Z/100	0.0036	0.017	0	2.443697	0.017
1.2 Z/100	0.00309	0.009	-0.006	2.510042	0.015
1 Z/100	0.00257	0.03	-0.005	2.590067	0.035
0.8 Z/100	0.00206	0.053	-0.007	2.686133	0.06
0.7 Z/100	0.0018	0.067	-0.008	2.744727	0.075
0.6 Z/100	0.00154	0.089	-0.001	2.812479	0.09
0.4 Z/100	0.00103	0.123	-0.006	2.987163	0.129
0.3 Z Z/100	0.000771	0.152	-0.007	3.112946	0.159
0.2 Z/100	0.000514	0.2	-0.005	3.289037	0.205
0.18 Z/100	0.000463	0.208	-0.007	3.334419	0.215
0.16 Z/100	0.000411	0.22	-0.004	3.386158	0.224
1.4 Z/1000	0.00036	0.223	-0.007	3.443697	0.23
1.0 Z/1000	0.000257	0.23	-0.005	3.590067	0.235
0.8 Z/1000	0.000206	0.236	-0.004	3.686133	0.24
0.6 Z/1000	0.000154	0.244	-0.001	3.812479	0.245
0.4 Z/1000	0.000103	0.243	-0.007	3.987163	0.025
0.2 Z/1000	5.14E-05	0.251	-0.005	4.289037	0.256

Note: In case of the most concentrated samples happened that their absorbance was lower than the absorbance of the blind samples. That is why the value of A was defined as 0.

Mosses

Dicranum polysetum:

dilution	mg/ml	sample	blind s	conc-log	difference
1.4 Z	2	0.52	0.533	-0.30103	0
1.4 Z/2	1	0.286	0.287	0	0
1 Z/2	0.714	0.211	0.22	0.146302	0
0.8 Z/2	0.571	0.2	0.169	0.243364	0.031
0.6 Z/2	0.429	0.2	0.136	0.367543	0.064
0.4 Z/2	0.286	0.199	0.084	0.543634	0.115
0.3 Z/2	0.214	0.216	0.061	0.669586	0.155
1.4 Z/10	0.2	0.205	0.038	0.69897	0.167
0.25 Z/2	0.179	0.227	0.038	0.747147	0.189
0.2 Z/2	0.143	0.25	0.035	0.844664	0.215
0.9 Z/10	0.129	0.247	0.028	0.88941	0.219
0.8 Z/10	0.114	0.244	0.024	0.943095	0.22
0.7 Z/10	0.1	0.249	0.025	1	0.224
0.6 Z/10	0.0857	0.248	0.023	1.067019	0.225
0.4 Z/10	0.0571	0.25	0.01	1.243364	0.24
0.3 Z/10	0.0429	0.249	0.006	1.367543	0.243
0.2 Z/10	0.0286	0.256	0.01	1.543634	0.246
0.1 Z/10	0.0143	0.273	0.01	1.8446644	0.263
0	0	0.265	0		0.265

Ceratodon purpureus:

dilution	mg/ml	sample	Blind s	Conc-log	difference
1.4 Z	2	0.372	0.391	-0.30103	0.001
1.4 Z/2	1	0.138	0.149	0	0
1 Z/2	0.714	0.147	0.118	0.146302	0.029
0.8 Z/2	0.571	0.163	0.078	0.243364	0.085
0.6 Z/2	0.429	0.199	0.054	0.367543	0.145
0.4 Z/2	0.286	0.227	0.033	0.543634	0.194
0.3 Z/2	0.214	0.229	0.02	0.669586	0.209
1.4 Z/10	0.2	0.23	0.019	0.669586	0.211
0.25 Z/2	0.179	0.237	0.022	0.69897	0.215
0.2 Z/2	0.143	0.236	0.012	0.747147	0.224
0.9 Z/10	0.129	0.236	0.012	0.844664	0.224
0.8 Z/10	0.114	0.235	0.007	0.943095	0.228
0.7 Z/10	0.1	0.24	0.006	1	0.234
0.6 Z/10	0.0857	0.239	0.005	1.067019	0.234
0.4 Z/10	0.0571	0.247	0.007	1.243364	0.24
0.3 Z/10	0.0429	0.248	0.002	1.367543	0.246

0.2 Z/10	0.0286	0.248	0	1.543634	0.248
0.1 Z/10	0.0143	0.257	0.002	1.844664	0.255
0	0	0.265	0		0.265

Dicranum scoparium:

dilution	mg/ml	sample	Blind s	Conc-log	difference
1.4 Z	2	0.526	0.525	-0.30103	0.001
1.4 Z/2	1	0.248	0.252	0	0
1 Z/2	0.714	0.202	0.181	0.146302	0.021
0.8 Z/2	0.571	0.2	0.15	0.243364	0.05
0.6 Z/2	0.429	0.192	0.111	0.367543	0.091
0.4 Z/2	0.286	0.204	0.074	0.543634	0.13
0.3 Z/2	0.214	0.211	0.048	0.669586	0.163
1.4 Z/10	0.2	0.189	0.021	0.6987	0.179
0.25 Z/2	0.179	0.217	0.038	0.747147	0.198
0.2 Z/2	0.143	0.236	0.038	0.844664	0.206
0.9 Z/10	0.129	0.237	0.031	0.88941	0.219
0.8 Z/10	0.114	0.242	0.023	0.943095	0.223
0.7 Z/10	0.1	0.24	0.017	1	0.225
0.6 Z/10	0.0857	0.241	0.016	1.067019	0.238
0.4 Z/10	0.0571	0.249	0.011	1.243364	0.245
0.3 Z/10	0.0429	0.247	0.002	1.367543	0.254
0.2 Z/10	0.0286	0.254	0	1.543634	0.259
0.1 Z/10	0.0143	0.26	0.001	1.844664	0.265

Leucobryum glaucum:

dilution	mg/ml	sample	bind s	Conc-log	difference
1.4 Z	2	0.838	0.876	-0.30103	0
1.4 Z/2	1	0.432	0.443	0	0
1 Z/2	0.714	0.312	0.315	0.146302	0
0.8 Z/2	0.571	0.226	0.244	0.243364	0
0.6 Z/2	0.429	0.219	0.179	0.367543	0.04
0.4 Z/2	0.286	0.21	0.119	0.543634	0.091
0.3 Z/2	0.214	0.214	0.088	0.669586	0.126
1.4 Z/10	0.2	0.2	0.066	0.6987	0.134
0.25 Z/2	0.179	0.2	0.055	0.747147	0.145
0.2 Z/2	0.143	0.214	0.049	0.844664	0.165
0.9 Z/10	0.129	0.213	0.037	0.88941	0.175
0.8 Z/10	0.114	0.226	0.037	0.943095	0.189
0.7 Z/10	0.1	0.241	0.036	1	0.205
0.6 Z/10	0.0857	0.249	0.034	1.067019	0.215
0.4 Z/10	0.0571	0.244	0.017	1.243364	0.227
0.3 Z/10	0.0429	0.247	0.015	1.367543	0.232

0.2 Z/10	0.0286	0.247	0.004	1.543634	0.243
0.1 Z/10	0.0143	0.267	0.005	1.844664	0.262
0	0	0.265	0		0.265

Mnium marginatum:

dilution	mg/ml	sample	Blind s	Conc-log	difference
1.4 Z	2	0.989	1.042	-0.30103	0
1.4 Z/2	1	0.512	0.519	0	0
1 Z/2	0.714	0.365	0.388	0.146302	0
0.8 Z/2	0.571	0.301	0.302	0.243364	0
0.6 Z/2	0.429	0.247	0.226	0.367543	0.021
0.4 Z/2	0.286	0.214	0.151	0.543634	0.063
0.3 Z/2	0.214	0.214	0.105	0.669586	0.109
1.4 Z/10	0.2	0.193	0.076	0.6987	0.117
0.25 Z/2	0.179	0.2	0.07	0.747147	0.13
0.2 Z/2	0.143	0.216	0.06	0.844664	0.156
0.9 Z/10	0.129	0.22	0.053	0.88941	0.167
0.8 Z/10	0.114	0.226	0.05	0.943095	0.176
0.7 Z/10	0.1	0.225	0.038	1	0.187
0.6 Z/10	0.0857	0.236	0.035	1.067019	0.201
0.4 Z/10	0.0571	0.26	0.027	1.243364	0.233
0.3 Z/10	0.0429	0.26	0.02	1.367543	0.24
0.2 Z/10	0.0286	0.251	0.005	1.543634	0.246
0.1 Z/10	0.0143	0.257	0.006	1.844664	0.251
0	0	0.265			0.265

Fenton´s reaction

Caffeic acid: NS

dilution	mg/ml	sample	blind s	conc-log	difference
0.5 Z	1.44	0.108	0.026	-0.158362	0.082
0.3 Z	0.864	0.145	0.015	0.063486	0.13
0.5 Z/4	0.36	0.222	0.013	0.443697	0.209
0.4 Z/4	0.288	0.244	0.014	0.540608	0.23
0.3 Z/4	0.216	0.268	0.01	0.665546	0.258
0.2 Z/4	0.144	0.293	0.011	0.841638	0.282
0.1 Z/4	0.072	0.355	0.011	1.142668	0.344
0.5 Z/40	0.036	0.421	0.01	1.443697	0.411
0.4 Z/40	0.0288	0.433	0.007	1.540608	0.426
0.3 Z/40	0.0216	0.467	0.007	1.665546	0.46
0.2 Z/40	0.0144	0.507	0.006	1.841638	0.501

0.1 Z/40	0.0072	0.574	0.009	2.142668	0.565
0.5 Z/400	0.0036	0.652	0.01	2.443697	0.642
0.4 Z/400	0.00288	0.667	0.006	2.540608	0.661
0.3 Z/400	0.00216	0.714	0.005	2.665546	0.709
0.2 Z/400	0.00144	0.759	0.006	2.841638	0.753
0.1 Z/400	0.00072	0.78	0.006	3.142668	0.774
0		0.87	0.015		0.855

Caffeic acid: S

dilution	mg/ml	sample	blind s	conc-log	difference
0.5 Z	1.44	0.022	0.019	-0.158362	0.003
0.3 Z	0.864	0.03	0.015	0.063486	0.015
0.5 Z/4	0.36	0.052	0.013	0.443697	0.039
0.4 Z/4	0.288	0.055	0.011	0.540608	0.044
0.3 Z/4	0.216	0.061	0.012	0.665546	0.049
0.2 Z/4	0.144	0.062	0.01	0.841638	0.052
0.1 Z/4	0.072	0.071	0.009	1.142668	0.062
0.5 Z/40	0.036	0.099	0.011	1.443697	0.088
0.4 Z/40	0.0288	0.122	0.009	1.540608	0.113
0.3 Z/40	0.0216	0.154	0.007	1.665546	0.147
0.2 Z/40	0.0144	0.219	0.008	1.841638	0.211
0.1 Z/40	0.0072	0.339	0.007	2.142668	0.332
0.5 Z/400	0.0036	0.483	0.008	2.443697	0.475
0.4 Z/400	0.00288	0.553	0.006	2.540608	0.547
0.3 Z/400	0.00216	0.642	0.006	2.665546	0.636
0.2 Z/400	0.00144	0.655	0.007	2.841638	0.648
0.1 Z/400	0.00072	0.672	0.006	3.142668	0.666
0		0.619	0.015		0.604

Mosses

Dicranum polysetum: NS

mg/ml	sample	blind s	conc-log	difference
2	0.649	0.102	-0.30103	0.547
1	0.755	0.062	0	0.693
0.5	0.828	0.041	0.30103	0.787
0.2	0.825	0.03	0.69897	0.795
0.1	0.843	0.021	1	0.822
0.05	0.88	0.016	1.30103	0.864
0	0.768	0.015		0.753

Dicranum polysetum: S

mg/ml	sample	blind s	conc-log	difference
3	0.278	0.15	-0.477121	0.128
2	0.412	0.11	-0.30103	0.302
1	0.515	0.054	0	0.461
0.5	0.596	0.035	0.30103	0.561
0.2	0.622	0.02	0.69897	0.602
0.1	0.652	0.016	1	0.636
0.05	0.672	0.013	1.30103	0.659
0	0.624	0.121		0.603

Ceratodon purpureus: NS

mg/ml	sample	blind s	conc-log	difference
2	0.729	0.06	-0.30103	0.669
1	0.777	0.036	0	0.741
0.5	0.836	0.037	0.30103	0.799
0.2	0.854	0.034	0.69897	0.82
0.1	0.851	0.013	1	0.838
0.05	0.866	0.017	1.30103	0.849
0	0.875	0.019		0.856

Ceratodon purpureus : S

mg/ml	sample	blind s	conc-log	difference
3	0.351	0.08	-0.477121	0.271
2	0.417	0.67	-0.30103	0.35
1	0.574	0.036	0	0.538
0.5	0.603	0.025	0.30103	0.578
0.2	0.651	0.017	0.69897	0.634
0.1	0.751	0.019	1	0.732
0.05	0.791	0.014	1.30103	0.777
0	0.513	0.013		0.5

Dicranum scoparium : NS

mg/ml	sample	blind s	conc-log	difference
2	0.619	0.116	-0.30103	0.503
1	0.638	0.067	0	0.571
0.5	0.672	0.049	0.30103	0.623
0.2	0.738	0.037	0.69897	0.701
0.1	0.768	0.022	1	0.746
0.05	0.801	0.017	1.30103	0.784
0	0.754	0.016		0.738

Dicranum scoparium : S

mg/ml	sample	blind s	conc-log	difference
3	0.265	0.12	-0.477121	0.145
2	0.27	0.104	-0.30103	0.166
1	0.287	0.046	0	0.241
0.5	0.399	0.038	0.30103	0.361
0.2	0.561	0.031	0.69897	0.53
0.1	0.648	0.025	1	0.623
0.05	0.65	0.014	1.30103	0.636
0	0.522	0.002		0.52

Leucobryum glaucum : NS

mg/ml	sample	blind s	conc-log	difference
2	0.361	0.145	-0.30103	0.216
1	0.533	0.08	0	0.453
0.5	0.763	0.06	0.30103	0.703
0.2	0.802	0.03	0.69897	0.772
0.1	0.814	0.018	1	0.796
0.05	0.849	0.012	1.30103	0.837
0	0.865	0.005		0.86

Leucobryum glaucum : S

mg/ml	sample	blind s	conc-log	difference
2	0.471	0.151	-0.30103	0.32
1	0.471	0.083	0	0.388
0.5	0.486	0.039	0.30103	0.447
0.2	0.537	0.025	0.69897	0.512
0.1	0.526	0.018	1	0.508
0.05	0.538	0.015	1.30103	0.523
0	0.536	0.005		0.531

Mnium marginatum : NS

mg/ml	sample	blind s	conc-log	difference
3	0.569	0.2	-0.477121	0.369
2	0.544	0.144	-0.30103	0.4
1	0.542	0.096	0	0.446
0.5	0.563	0.068	0.30103	0.495
0.2	0.574	0.03	0.69897	0.544
0.1	0.569	0.025	1	0.544
0.05	0.58	0.017	1.30103	0.563
0	0.643	0.025		0.618

Mnium marginatum : S

mg/ml	sample	blind s	conc-log	difference
3	0.294	0.219	-0.477121	0.075
2	0.234	0.155	-0.30103	0.079
1	0.19	0.073	0	0.117
0.5	0.286	0.036	0.30103	0.25
0.2	0.407	0.028	0.69897	0.379
0.1	0.446	0.021	1	0.425
0.05	0.456	0.016	1.30103	0.44
0	0.463	0.011		0.452

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